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The Influence of Acid Rain on Mycorrhizae: The Roles of Nitrate and Sulfate Ions and Indole Acetic Acid in the Development of *Pisolithus Tinctorius* on *Pinus Taeda L.*

Isaac M. Andrews

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THE INFLUENCE OF ACID RAIN ON MYCORRHIZAE: THE ROLES OF NITRATE AND SULFATE IONS AND INDOLE ACETIC ACID IN THE DEVELOPMENT OF *PISOLITHUS TINCTORIUS* ON *PINUS TAEDA* L.

A thesis submitted by

Isaac M. Andrews

B.A. 1978, Virginia Polytechnic Institute & State University

M.S. 1980, Lawrence University

in partial fulfillment of the requirements
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SUMMARY

Loblolly pine seedlings (Pinus taeda L.), which had been successfully inoculated with the fungus Pisolithus tinctorius to form mycorrhizae, were watered over a nine-month period with several types of low pH solutions, including two treatments that simulated acid rain. All solutions were applied to the soil. From these treatments it was learned that a simulated acid rain treatment at a pH of 4 approached the limit of tolerance for the mycorrhizal association. Solutions of sulfuric acid at pH 4 and pH 5, and nitric acid at pH 4, adversely influenced mycorrhizal development. The pH 5 acid rain treatment, because of an apparent interaction between the nitrogen and sulfur, had no adverse influence on mycorrhizal development. A change in pH per se, as tested with weak solutions of hydrochloric acid, had no effect on mycorrhizal development.

The growth hormone, indole acetic acid (IAA), is reported to be a molecular agent responsible for the formation of mycorrhizae. IAA is supplied to the tree by the fungal component of mycorrhizae. In this investigation, roots with extensive mycorrhization showed no significant difference in levels of free indole acetic acid when compared with roots having little or no apparent mycorrhizae. Levels of IAA in mycorrhizae were either cyclical or were inaccessible to the experimental analysis due to molecular binding, and thus not correlated with mycorrhizal development.

The nitrogen component of acid rain produced a "fertilizer" effect on overall tree growth. The effect was reduced by the presence of sulfur. Levels of sulfur in seedling needles were shown to be negatively correlated with shoot fresh weight.

A reliable method for measuring levels of free IAA in roots was developed. The procedure was also suitable for measuring IAA in tissue culture systems.

INTRODUCTION

Mycorrhiza is a word coined from two Latin cognates: myco-, meaning fungus or mushroom, and rhiza, or root. Literally then, a mycorrhiza is a "root-fungus," but such a simplified description is somewhat misleading. It is more accurate to view a mycorrhiza as a distinct anatomical structure with its own physiological and metabolic state independent of either the root or the fungus.

Mycorrhizae are widely distributed in the plant kingdom, especially among herbaceous and woody species. For gymnosperms alone there are nearly a thousand known fungal associations. A mycorrhizal fungus is not detrimental to a tree; on the contrary, it is the cornerstone of a complex symbiotic relationship which benefits both the tree and the fungus. The benefits of mycorrhizae are numerous and have been well summarized by Slankis (1). A typical physiological response of a tree root to a mycorrhizal fungus is a swelling and forking of the root (Fig. 1). This serves to increase the root's surface area, which in turn

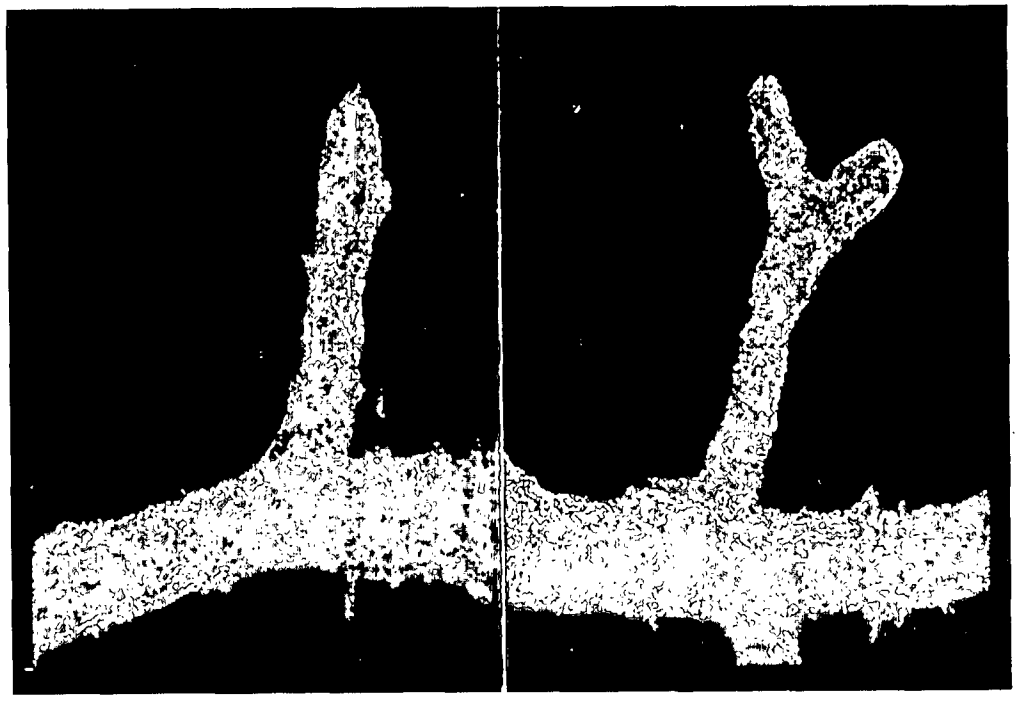

Figure 1. Computer enhanced color image of a normal root and a mycorrhiza. Notice the forking of the root tip in the image on the right.

increases the moisture and nutrient uptake of the root. This process is reversible, and researchers have shown that levels of certain nutrients, such as phosphorous (2) or nitrogen (2,3), can cause the mycorrhiza to disappear, leaving a root with its normal appearance.

It is natural to assume that some agent produced by the fungus is responsible for the physical changes which occur in the root upon infection. Analysis of fungal exudates (4) and their effects on excised root cultures (5,6) have shown that the agent is a growth regulating hormone, specifically indole acetic acid (IAA). Figure 2 shows the structure of an IAA molecule. IAA is just one of the many growth hormones which occur in nature. The indole structure is the basic moiety for many auxins but not for all growth regulating substances. For example, cytokinins and giberellins exist without this basic chemical skeleton. There have been studies which show that high nitrogen levels decrease the levels of fungal auxins in culture (7,8).

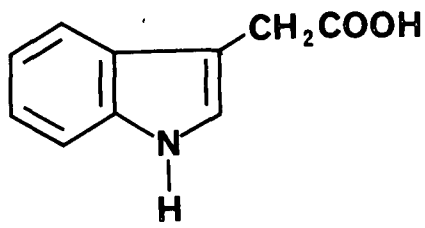

Figure 2. Molecular structure of IAA.

Nitrogen is an important component of acid rain. In the form of the nitrate ion, it makes up 30-35% of the anionic species in acid precipitation (9,10). Sulfur is the major component of acid rain, with the sulfate ion composing from 65-70% of the ionic species. In some parts of the country, chloride ion is present, but only in amounts of 5% or less (11).

Normal precipitation would be considered acidic, since it has a pH value of 5.6. This would be the pH expected from atmospheric moisture in equilibrium with carbon dioxide, to form carbonic acid (Fig. 3). Sulfur dioxides, as well as nitrous and nitric oxides released from man-made and natural sources also react with atmospheric moisture to produce sulfuric and nitric acid (Fig. 4 and 5), which can further lower the precipitation pH. Current net emissions of sulfur and nitrogen oxides in the United States are estimated to be 27 and 22 million tons, respectively (11). Natural sources, such as volcanic eruptions, lightning, decomposition of organic matter and salt sprays from the ocean (to name a few) are significant contributors to acid precipitation.

(1) $CO_2 + H_2O \rightleftharpoons H_2CO_3$

(2) $H_2CO_3 \rightleftharpoons H^+ + HCO_3^-$

Figure 3. Equilibrium of carbon dioxide with water (12).

(a) $N_2 + O_2 \longrightarrow 2\,NO$

(b) $2\,NO + O_2 \longrightarrow 2\,NO_2$

(c) $2\,NO_2 + H_2O \longrightarrow HNO_2 + HNO_3$

Figure 4. Reactions of nitrogen (13).

(a) Formation of nitric oxide at combustion temperatures.

(b) Oxidation of NO to nitrogen dioxide in either the combustion zone or atmosphere.

(c) Hydration of NO_2 to form both nitrous and nitric acids.

(a) $S + O_2 \longrightarrow SO_2$

(b) $SO_2 + H_2O \longrightarrow H_2SO_3$

(c) $H_2SO_3 + \frac{1}{2}O_2 \longrightarrow H_2SO_4$

Figure 5. Reactions of sulfur (13).

(a) Formation of sulfur dioxide at combustion temperatures.

(b) Hydrolysis of SO_2 to form sulfurous acid.

(c) Oxidation of H_2SO_3 to form sulfuric acid.

The various effects of lower precipitation pH's on the ecosphere are rapidly coming to light. The effects on lakes and streams and the aquatic life within are well documented, with one unfortunate consequence being the total loss of fish populations. The effects on forest sites are starting to surface after several years of studies and assessments; yet, in some areas, little if any information is available. One of these areas is the effects of acid rain on the symbiotic relationship between mycorrhizal fungi and tree roots, the focus of this thesis.

THESIS OBJECTIVE

Before stating the thesis, it is perhaps best to review three of the facts presented in the previous section: (1) IAA produces mycorrhizal characteristics in roots, (2) critical levels of nitrogen cause a reversion of mycorrhizal characteristics, (3) acid rain contains nitrogen compounds. The thesis statement was synthesized from a combination of these three facts: there is sufficient nitrogen in acid rain to disrupt IAA synthesis by mycorrhizal fungi and cause the mycorrhizae to disappear.

In this thesis, acid rain was formulated in the laboratory and poured into pots which contained loblolly pine seedlings (Pinus taeda L.) infected with the mycorrhizal fungus Pisolithus tinctorius. The anionic components of the acid rain solution were singled out and were also applied in equal measures to the seedlings in order to determine if there were any synergistic effects between the sulfate and nitrate ions. This method also permitted the study of the effects of the individual ions. In addition to the sulfate and nitrate solutions, acid precipitation was formulated with hydrochloric acid in an effort to understand the effects of changing pH on the system. Finally, a control solution representing "normal" rainfall was applied to give a performance baseline. Changes in the degree of mycorrhization were assessed and the levels of free IAA in the roots were measured at the completion of the acid rain treatments, along with standard tree performance parameters.

It was hoped that this thesis would shed more light on the role of IAA and nitrogen in the forming of mycorrhizae. The biochemical mechanisms responsible for mycorrhizal formation have not been completely elucidated. The information gleaned from this study may be of benefit to foresters, too, especially those

who routinely inoculate seedlings with mycorrhizal fungi for outplanting in adverse sites. Deleterious effects of acidic precipitation on mycorrhizae might persuade them to review their practice in light of their local climatological conditions.

LITERATURE REVIEW

The close association of fungi with trees has been known for centuries. This conclusion is drawn from the fact that there exist common names for mushrooms in the Russian and German languages which include the names of the trees under which they are found: dubovik (oak mushroom, Boletus luridus) and Larchenmilchling (larch lactarius, Lactarius porninsis) (14). Perhaps the earliest reference to mycorrhizae in scientific literature is found in the December 31, 1885 issue of Nature. A reporter at the annual meeting of the Association of German Naturalists and Physicians recounts a discussion among some botanists who had recently discovered the fact that a considerable number of forest trees "do not draw their nourishment directly from the soil, but through the medium of an investing [sic] layer of fungus-mycelium, to which B. Frank gives the name of Mycorhiza [sic]" (15). Dr. Frank, the article points out, "regards the phenomenon as an example of symbiosis."

Interest in the role fungi play in the development of mycorrhizae did not develop until the early 1930's. At that time, Nielsen (16) showed that fungi in pure culture produce a growth regulating substance which he termed "rhizopin." Following up this study, Thimann (17) determined the growth regulator to be IAA. Shortly thereafter, Slankis (5) tested some fungal exudates on excised root cultures of Pinus sylvestris L. and found that they produced morphological changes characteristic of mycorrhizae. In a similar study, Turner (6) observed that fungal exudates from a variety of Boletus fungi also produced mycorrhizal characteristics in excised Pinus sylvestris L. root cultures. An analysis of Boletus and Amanita exudates by Ulrich (4) showed the presence of several growth regulating substances, chiefly IAA. That IAA is directly responsible for root development was demonstrated by Slankis (18). Using IAA and

other growth regulating substances, he observed that these compounds have a marked effect on the development of isolated and intact pine roots, and that the growth response is concentration dependent: the more auxin applied, the more pronounced the mycorrhizal characteristics.

The response of root growth to IAA is regulated by nutrients, and somehow this effect is tied in with the action of the fungal component of mycorrhizae. Tomaszewski & Wojciechowska (7) and Moser (8) have tested fungal exudates in the presence of high levels of nitrogen and found that the production of IAA is diminished. Working directly with infected roots, Slankis (19) noticed significant structural changes in well-developed mycorrhizal roots after exposure to high levels of nitrogen. Previously swollen root apices had elongated, and the newly formed regions had become slender and covered with root hairs. Root sections revealed that the Hartig net, the internal manifestation of mycorrhizae, had fragmented. Mycorrhizal roots from the same source but treated with much lower levels of nitrogen retained their usual mycorrhizal appearance, and sections revealed an intact Hartig net.

Subsequent investigations have shown that not only nitrogen, but phosphorous as well, will inhibit the formation of mycorrhizae on pines. Marx and Barnett (20) and Marx et al. (2) found that significantly more mycorrhizae were formed on seedlings which had received low levels of N and P in the rooting medium than those in a medium with high levels of N and P. Johnson and Joiner (21) also observed reduced infection of mycorrhizae in rhododendron under high N levels. In contrast to these widely accepted views of the role of soil fertility on mycorrhizal formation, there are some who feel that excessive fertilization is not necessarily inhibitory to mycorrhizal formation, so long as a proper and full balance of nutrients are supplied (19).

In all of the studies concerning nitrogen and mycorrhizae, nitrogen was supplied to the experimental systems in some form other than nitric acid, the secondary component of acid rain. Investigations on the influence of acid rain on mycorrhizae are scarce, and the only known study to date examined only the role of sulfur. This was performed by Hung and Trappe (22) when they examined the growth variation of ten species of ectomycorrhizal fungi exposed to a pH range of 2-7. Liquid media were treated with sulfuric acid before inoculation with the mycorrhizal fungi. P. tinctorius was among the fungi tested, and the researchers found that a significantly higher mean mycelial dry weight per milliliter nutrient solution existed for the P. tinctorius at pH of 4 and 5. Since the purpose of the study was to gauge the tolerance of the fungi to acidic conditions, effects of sulfur on in vivo mycorrhizae were naturally excluded.

While the effects of acid rain on mycorrhizae have been largely overlooked, the impact of acid precipitation on conifers and coniferous sites has been assessed in several studies. Wood and Bormann (23) applied a 66:24:10 solution of sulfate/nitrate/chloride ions to month-old Pinus strobus L. seedlings for a period of 20 weeks. Foliar damage was observed at only the lowest pH (2.6), and each of the seedlings showed increased productivity as acidity increased. Leaching of magnesium and calcium cations steadily increased with rising acidity. Cole and Johnson (24) studied the effect of atmospheric sulfate additions to a Douglas-fir ecosystem in the state of Washington, where the precipitation pH frequently fell below 4. However, they observed that solutions collected at the forest floor and soil solutions at a depth of 4 cm did not at any time have a pH less than 5. They concluded that most of the annual incoming H ions were removed in the forest canopy. Cole and Johnson speculated on the role of increased sulfur addition, vis a vis cycling acceleration, but did not make definite conclusions

because of the lack of a suitable control site. Abrahamsen et al. (25) studied the effects of various factors of acidic precipitation on spruce and pine ecosystems. Like Wood and Bormann (23), they too found a reduction of base saturation of the study soil due to leaching of magnesium and calcium. They also found that germination of spruce seeds in acidified mineral soil was negatively affected when soil pH was 4 or less.

Overall, documentation for reduced forest growth has been rather unsuccessful. The major reasons have been explained by Einspahr (12), and include the fact that acid rain modifies forest soils very slowly. This would indicate that the reductions in base saturation due to Mg and Ca leaching may not be significant until decades have passed. Furthermore, nitrogen as a component of acid rain, is also the element most associated with improved tree performance. Obviously, the key to understanding the impact of acid precipitation lies in quantifying the amounts of man-made atmospheric elements deposited, and then correlating this with tree performance parameters. Controlled studies in laboratories and greenhouses reveal specific information which can be used as an indicator for real world behavior.

METHODS AND MATERIALS

SEEDLING PREPARATION

Seeds of Pinus taeda L. (Source: International Seed Co., Cullman County, Alabama; collected in 1977) were treated with 30% H_2O_2 for 60 minutes before planting. Approximately 3 seeds were planted in each section of a Spencer-Lemaire Rootrainer filled with a 1:1:1 mixture of sand/peat/vermiculite, after a method fashioned by Graham and Linderman (26). The mixture had previously been fumigated by treatment with a 500 mL methyl bromide bomb for 48 hours.

After germination the seedlings were allowed to develop in a greenhouse under sunlight plus sodium vapor lamps for a total photoperiod of 16 hours. The temperature varied between 22 and 32°C, depending on the season, and the seedlings were watered daily as needed. In those Rootrainer sections where germination was complete and where damping off did not occur, the seedlings were thinned down to one per section for a total of 1232.

INOCULUM PREPARATION

The fungus selected for use in this study was Pisolithus tinctorius, a well-known associate with loblolly pine. (Source: Donald Marx, Director, Institute for Mycorrhizal Research & Development, Athens, Georgia; isolated from a fruiting body under loblolly pine in September, 1980). Two hundred mL of modified Melin-Norkrans liquid medium (see Appendix I) were poured into each of 100 pint-sized Mason jars and autoclaved for 15 minutes at 120°C and 15 psi. Each jar was then inoculated with a small clump of P. tinctorius from stock cultures made from the isolate and growing on potato dextrose agar. The P. tinctorius was allowed to incubate for 2-1/2 months in the dark at 25°C.

INOCULATION OF SEEDLINGS

Two months after germination, the Rootrainers were opened one at a time and the soil brushed back to expose the roots. Then, 15 cc of the *P. tinctorius* inoculum were applied directly on the roots, giving a 10:1 ratio of soil to inoculum. The containers were then folded shut and returned to the flat from which they came. This procedure continued until the supply of *P. tinctorius* was exhausted, which happened after 784 seedlings were inoculated (some of the *P. tinctorius* had been lost to contamination or had failed to incubate).

The seedlings continued to grow under the previously specified conditions. However, a month after the inoculation, the seedlings began to appear spindly and yellowish. They were fertilized with a 2500 ppm solution of Peters 20-20-20 fertilizer, with 30 mL applied to each seedling. This was the only fertilizer treatment the seedlings received during the entire study.

SELECTION OF MYCORRHIZAL SEEDLINGS

Four months after inoculation, the Rootrainers were reopened and the roots of the individual seedlings were examined for mycorrhizal infection. The criteria used for assessing infection were (a) presence of mantle, (b) two or more bifurcated roots per lateral, and (c) discoloration of root (usually yellow).

The rate of successful infection of all inoculated seedlings was 48%, giving over four times as many mycorrhizal seedlings as needed. From this abundance it was possible to select a group of seedlings which appeared uniform in their extent of mycorrhization.

SOIL

The soil used in the study was a mixture of the A horizons of two forest soils indigenous to Wisconsin (near the communities of Clintonville and Eagle River). Elemental analysis revealed the soil to be high in nitrate-N (195 ppm) and low in P (less than 1 ppm). Levels of K, Ca, and Mg were acceptable (32, 174 and 56 ppm, respectively). Levels of sulfate-S were 20 lb/acre, and organic matter 33 tons/acre. The soil texture was a loamy sand (84% sand, 11% silt, 5% clay), and the soil had a pH 5.2. The cationic exchange capacity was approximately 3 meq/100 g soil.

GREENHOUSE SET-UP

Three mycorrhizal seedlings were planted into each of 32, 7-inch plastic pots which had been filled with soil up to 1 1/4-inch from the top. Twenty-seven of the pots were divided into nine groups which represented the nine types of acid rain treatment (eight treatments plus control) to be applied (Table I). These groups were arranged in a randomized block design on a greenhouse bench. No acid rain treatments were applied during the first month, in order to give the seedlings an opportunity to acclimatize. The few seedlings which died during this period were replaced with the seedlings from the remaining five pots not included in the study.

TABLE I

SIMULATED ACID RAIN TREATMENTS

Group	SN5	S5	N5	CL5	SN4	S4	N4	CL4	CON
Number of pots	3	3	3	3	3	3	3	3	3
pH	5.0	5.0	5.0	5.0	4.0	4.0	4.0	4.0	5.6
Prevalent ions	$SO_4^{=}$ NO_3^{-}	$SO_4^{=}$	NO_3^{-}	CL^{-}	$SO_4^{=}$ NO_3^{-}	$SO_4^{=}$	NO_3^{-}	CL^{-}	

The growing conditions were the same as previously described, with the exception that after one month the simulated acid rain treatments were initiated. At each application, the treatment amounts were measured into a beaker and poured directly onto the soil. During the following nine months, each pot received a total of 8 liters of the assigned acid rain treatment. For the pH 5 treatments, this amounted to a total of approximatley 5 lb/acre sulfur and 2 lb/acre nitrogen; for pH 4, 13.5 lb/acre sulfur and 4.5 lb/acre nitrogen. Due to a high moisture loss through evaporation, transpiration, and drainage, the trees also received additional deionized water as needed. Only deionized water was used on the control group.

FORMULATION OF SIMULATED ACID RAIN SOLUTIONS

The simulated acid rain solutions were made a batch at a time and were applied immediately after making. Four acid rain solutions were made for two different pH, 5.0 and 4.0. The solutions were

(1) acid rain composed of a 7:3 mixture of sulfuric and nitric acid (designated SN5 and SN4),

(2) acid rain composed of sulfuric acid only, equivalent to levels of sulfuric acid in (1), and adjusted to the same pH with 2N HCl (S5 and S4);

(3) acid rain composed of nitric acid only, equivalent to levels of nitric acid in (1) and adjusted to the same pH with 2N HCl (N5 and N4);

(4) acid rain composed of hydrochloric acid only and adjusted to same pH as (1), (2), and (3) (CL5 and CL4).

To make solution (1), concentrated sulfuric acid (98%) was diluted to the same concentration as nitric acid (70%), then the two acids were diluted 1:1000 with

distilled water. Seven mL of the sulfuric acid dilution was then mixed with 3 mL of the nitric acid dilution before pouring into a graduated burette. The dilute mixture was then titrated into 1000 mL of distilled water until the target pH was reached (either 5.0 or 4.0). pH measurements were made with an Orion Digital Ionalyzer No. 501. To make solutions (2) and (3), the volume of sulfuric/nitric acid mixture used to reach the desired pH was multiplied by the appropriate factor (0.7 for sulfuric and 0.3 for nitric) to determine the amounts of individual acids used in the formulations. Then these amounts were added to separate beakers filled with 1000 mL distilled H_2O and the pH further reduced to the target pH with 2N HCl added dropwise. Finally, to make solution (4), 2N HCl was added dropwise to two additional beakers filled with 1000 mL H_2O until the desired pH was reached.

In summary, this procedure gave 8 solutions: 4 at pH 5.0 and 4 at pH 4.0. At each pH, there was one acid rain solution composed of both sulfuric and nitric acids, one solution containing an equivalent amount of sulfuric acid only, one solution containing an equivalent amount of nitric acid only, and one solution with neither ion, but containing hydrochloric acid. The ninth solution was simply 1000 mL deionized water for use in the control treatment.

HARVESTING OF SEEDLINGS

Nine months after the initiation of the acid rain treatments, the trees were harvested. Shoot heights were measured before the soil was removed from the pot and the roots examined for extent of mycorrhization. The observed roots were assigned to one of four groups depending upon the degree of mycorrhizal infection: Group 1 - 0-24%, Group 2 - 25-39%, Group 3 - 50-74%, Group 4 - greater than 75%. Percent infection was determined by examining 3 random root

sections and counting the number of mycorrhizal structures per 10 laterals. The 3 counts were averaged and the result was compared with a visual estimate based on overall appearance. If the average and the estimate differed by 25% or more, the first value was recorded and the process repeated for three more random root sections. In addition, comments were recorded to describe extreme cases of infection (or noninfection). These data were used to create a Mycorrhizae Index (M-Index), which gives an indication of the relative degree of infection among the experimental seedlings (Appendix II).

After the assessment for the extent of mycorrhization, the roots were rinsed under cold running tap water to remove dirt. They were then blotted dry with paper towels and separated from the shoot at a point just above the first lateral. Root and shoot weights were recorded, then the roots were sealed in plastic bags and stored at -20°C. The needles were stripped from the shoots and dried at 105°C for 24 hours. Then, using a 40 mesh screen, the needles were ground in a Wiley mill and were sealed in coin envelopes. They were shipped to the Soil & Plant Analysis Laboratory of the University of Wisconsin Extension for elemental analysis (Appendix III). Three samples were run for each treatment.

EXTRACTION OF IAA FROM ROOTS

The extraction and processing of IAA from the roots involves several modifications of the extraction procedure utilized by Caruso et al. (27) and outlined by McDougal and Hillman (28). A flow diagram in Fig. 6 summarizes the procedure used in this study. The roots were removed from the freezer and allowed to thaw for one hour at room temperature. Three mL of methanol per gram of fresh root

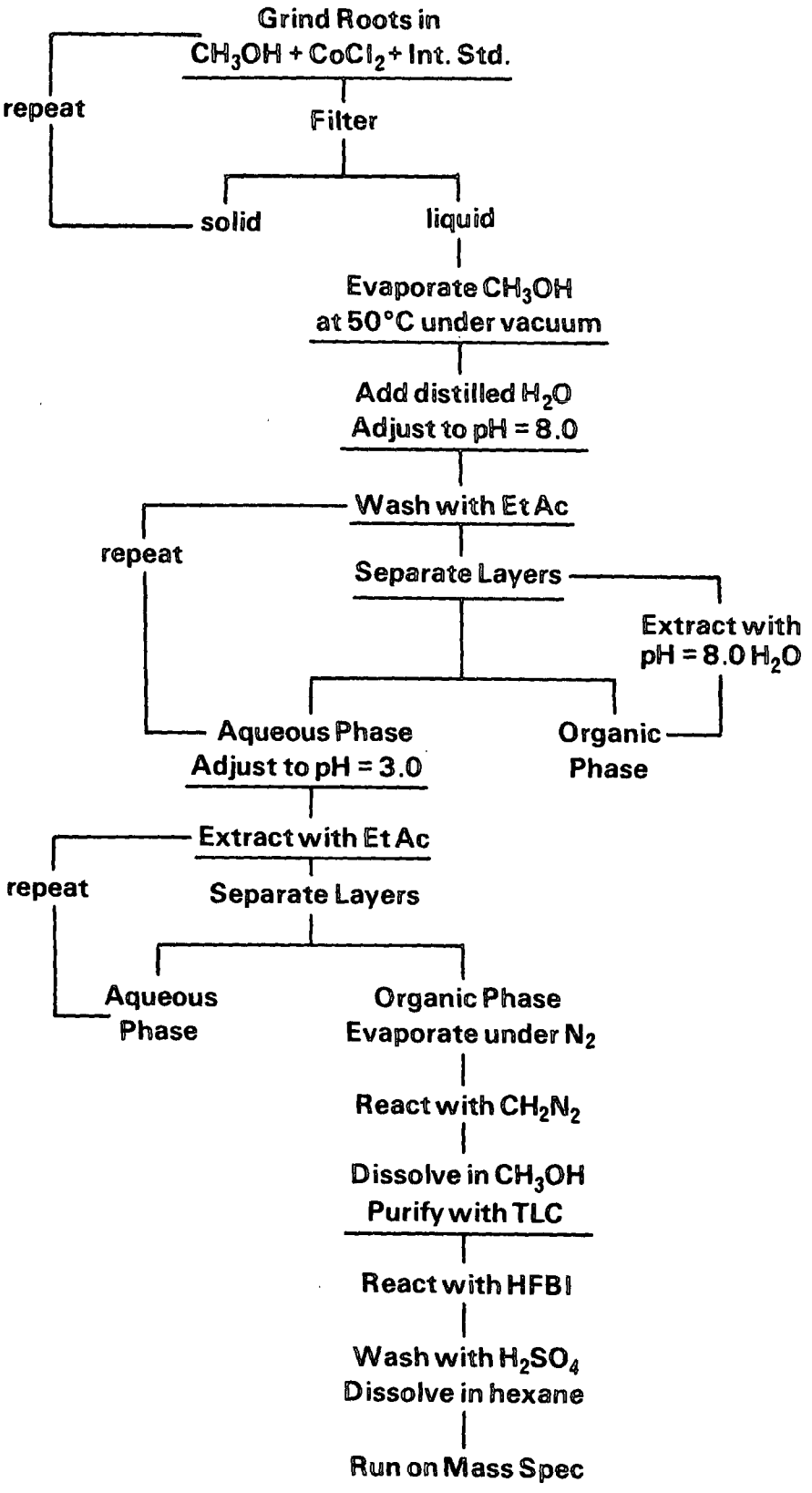

Figure 6. Flow diagram for IAA extraction method.

weight were used to extract IAA from the sample. In addition, the methanol was made 0.1M with cobaltous chloride, as $CoCl_2$ has been reported to prevent degradation of free IAA for up to 24 hours (29).

After the stock solution of $CH_3OH/CoCl_2$ was prepared, 10 µL of 1-^{14}C-IAA was added as internal standard (Source: Amersham, Arlington Hgts., IL; specific activity = 59 mCi/mmol, radiochemical purity = 97%). Three 1 mL aliquots were withdrawn and to each were added 10 mL of Scintisol (Interex Corp.) counting cocktail. Radioactivity was determined in a Beckman scintillation counter, Model DPM-100. Quench curves for the cocktails were those determined by Monroe (30) (Appendix IV).

The roots were cut into 1 cm pieces and placed in a mortar. One half of the methanol was poured over the roots, and the roots were allowed to soak in the solution for one hour at room temperature. They were then ground with a pestle for 15 minutes before the liquid was decanted into a filter funnel (under vacuum) fitted with Whatman No. 1 filter paper. The procedure was then repeated for the remaining half of the methanol solution, except that the soaking time was reduced to 15 minutes. The combined extracts were then evaporated in a Rotovap under vacuum at 50°C until all the methanol was removed as determined by a sudden change in the evaporation rate of the extract.

PARTITIONING OF EXTRACT

The concentrated extract was then adjusted to pH 8.0 followed with a pH meter with a 30% sodium hydroxide solution and poured into a separatory funnel. An equal volume of ethyl acetate was added, and the mixture was shaken. The organic fraction was saved, and a fresh portion of ethyl acetate was added and the

extract was repartitioned. The combined organic fractions were then poured into a clean separatory funnel and were mixed with two portions of distilled water which had been adjusted to pH 8.0 with the 30% NaOH. The amount of H_2O/NaOH used in each partitioning of the organic fraction equaled the volume of the combined organic fractions. The combined aqueous fractions from this step were added to the aqueous fraction remaining after the first two treatments with ethyl acetate.

The aqueous extract solution was then adjusted to pH = 3.0 with 2N HCl. The aqueous phase was then partitioned twice with an equivalent volume of ethyl acetate, and the combined organic fractions were reduced in volume in the Rotovap. The concentrate was transferred to a 3 mL Reactivial (Pierce Chemical Co.) and evaporated to dryness under a steady stream of nitrogen.

FIRST DERIVATIZATION OF EXTRACT

The IAA sample was then carried through the first of a two step derivatization process patterned after Rivier and Pilet (31). The dried extract received 2 mL of a cool diazomethane solution (Appendix V). The reaction was carried out at room temperature for 5 minutes, then the Reactivial was sealed and stored at -20°C until ready for further processing. The reaction product is shown in Fig. 7.

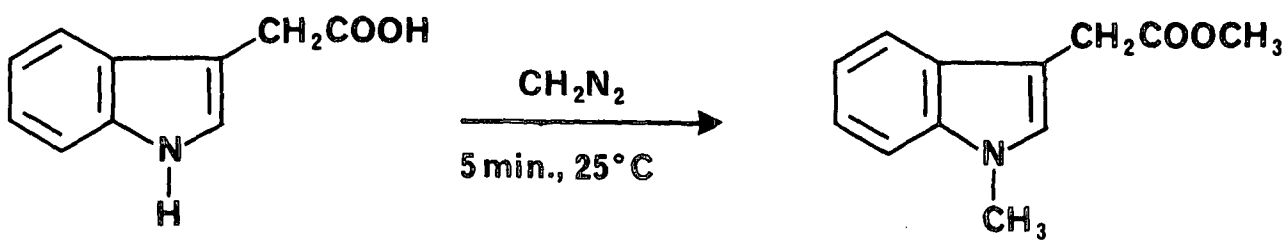

Figure 7. Reaction of IAA with diazomethane.

PURIFICATION OF DERIVATIZED PRODUCT BY TLC

The derivatized product was evaporated and redissolved in a working volume of CH_3OH before spotting on Whatman K6 silica gel thin layer chromatography plates with a layer thickness of 250 μ. The size of the plates was 5 x 20 cm, and in some instances it was necessary to use 2 or even 3 plates to prevent overloading. Addition of methanol and spotting continued until the insides of the Reactivials were free from deposits, and addition of any more methanol produced only a colorless solution.

The plates were developed in a 70:25:5 mixture of chloroform/methanol/acetic acid along with a standard plate spotted with a methylated standard IAA sample. Developing time usually lasted 1-1/2 hours, after which time all plates were removed. The standard plate was sprayed with van Urk's reagent (32) and the R_f value was calculated. This value was used to locate the methylated IAA extract on the sample plates. The R_f value from the standard varied from time to time between 0.92-0.96, and the sample plates were scraped in a range of $\pm$ 0.02 of the standard R_f. The silica gel was washed 3 times with 2 1/2 mL CH_3OH and centrifuged after each washing. The supernatant was decanted each time into a 3-mL Reactivial and evaporated to dryness under N_2.

SECOND DERIVATIZATION OF EXTRACT

After completion of the chromatography step, the dried extract was treated with 100 μL of heptafluorobutyrylimidazole (HFBI) (Source: Pierce Chemical Co.) and sealed in the Reactivial. The reaction was carried out at 80°C for 2 hours. The product had the molecular structure as shown in Fig. 8.

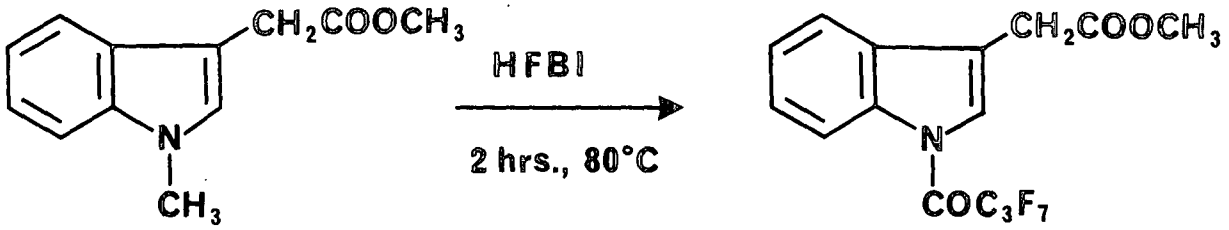

Figure 8. Derivatization of methylated IAA with HFBI.

After cooling, the Reactivial was opened and the sample washed in the vial with 2 mL of 1N H_2SO4, which was then extracted with 1 mL hexane. The sample was stored at -20°C until the aqueous layer froze, then the hexane layer was drawn off and transferred to a 1 mL Reactivial, where it was evaporated to dryness under N_2. An additional 1 mL of hexane was added to the sulfuric acid wash, and the process was repeated. The final volume of the sample was kept at 1 mL, and the sample was stored at -20°C until ready for analysis.

MASS SPECTRA OF THE IAA DERIVATIVE

All samples were run on a Hewlett-Packard Mass Spectrometer, Model 5985, equipped with an H-P Gas Chromatograph, Model 5840A. Five µL were injected for each sample run. A 6 ft. x 2 mm i.d. glass column packed with 3% OV-17 on 100/120 Gas-Chrom Q was used, and the carrier gas was helium. The machine was programmed in the selective ion monitoring mode (SIM) and the peaks monitored were 387, the molecular ion of the internal standard, 385, the molecular ion of the sample IAA derivative, and 326, the base peak. The selected ion chromatograms of a known IAA derivative is shown in Fig. 9a, with that of a typical sample in 9b. The 326/385 peak area ratio was used as a check for internal consistency. If this ratio differed

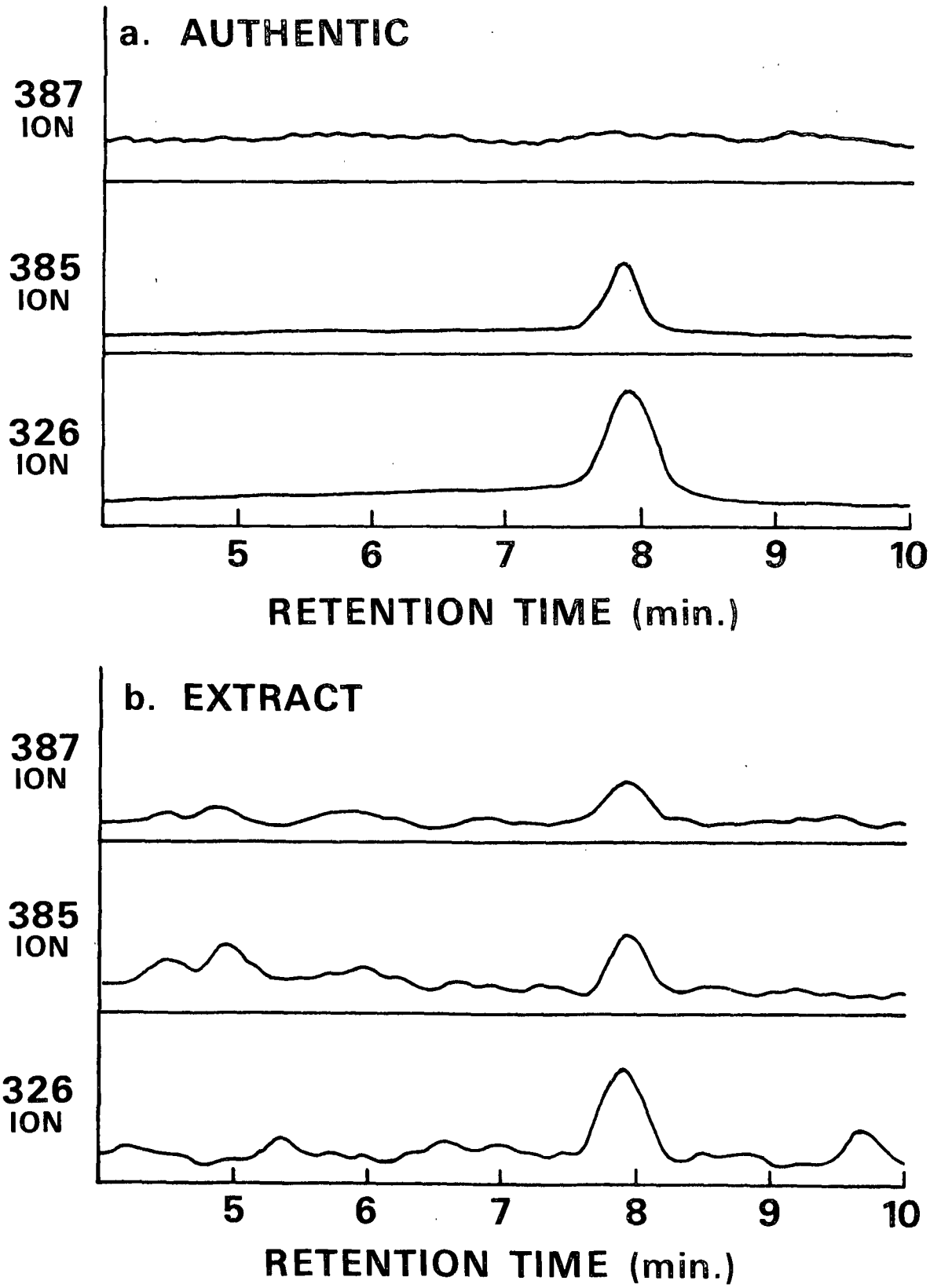

Figure 9. (a) Selected ion chromatogram of IAA derivative (authentic).
(b) Selected ion chromatogram of extract derivative.

significantly from the average value, the run was repeated. The appearance of the 387 peak with the 385 peak, in conjunction with the retention time of the known IAA derivative, gave sufficient verification of the presence of IAA in the sample.

CALIBRATION CURVES

A stock solution of IAA was prepared and diluted serially to 40 ppm. The IAA was then carried through the same 2 derivatization steps as a regular sample. No TLC or sulfuric acid wash was carried out. Because the fine tuning of the mass spectrometer varied from day to day, a calibration curve was constructed whenever a set of samples was run. Injection volumes and dilutions were manipulated to give a peak area higher than the highest one recorded for the samples that day, and to give a peak area lower than the lowest one also recorded for the day's run. At least two points were generated for the areas in between. Curves were constructed using a least squares analysis on Texas Instrument TI-55-II hand calculator. The correlation coefficients for each curve were either 0.99 or 1.00.

RECOVERY OF INTERNAL STANDARD

When all the runs had been completed on a given sample, a 0.1 mL sample was withdrawn from the remaining hexane containing the IAA derivative and the internal standard. A cocktail was made with 10 mL of Scintisol, and three counts were performed. The average of the three counts was used to calculate the percentage recovery of the internal standard.

Before the final sulfuric acid wash, the percent recovery of the internal standard averaged around 60%. However, experience in the development of the extraction procedure showed that some unwashed samples contained contaminants

which occluded the 385 and 326 peaks. Although washing reduced the average recovery to 20%, it was felt that this loss of recovery was worth the gain in peak clarity. Even though the average recovery was 20%, only 10% loss could not be accounted for through scintillation counts of the various discarded fractions, including the waste sulfuric acid wash. The procedure for calculating the amount of IAA in the root sample is demonstrated in Appendix VI.

STATISTICAL ANALYSES

A one-way analysis of variance was calculated for the results from the nine treatments using a software program suitable for an Apple II personal computer. Probabilities were automatically computed and printed out. Treatment effects were considered significant when $P = 0.05$, and highly significant when $P = 0.01$. Correlation coefficients were also determined on the Apple II, and levels of significance were tested against values tabulated by Snedecor (33). The same program was used to perform ANOVA calculations for a three-factor (S, N, and pH) factoral arrangement of the data, which results whenever the control treatment and block effects are dropped (block effects must be insignificant to be dropped).

When ANOVA calculations were performed and there were statistical cells where the sums of squares of deviations were extremely high, outlier tests were performed on the data to determine if the deviants could be dropped. The test follows that of the TAPPI Provisional Test Method T 1205 ts-63 (revised October 1979), and again levels were chosen at $P = 0.05$ and $P = 0.01$. Calculation of estimators to replace the deviant data points was performed according to the method by Snedecor (33); the procedure is duplicated in Appendix VII. Snedecor was also the source of the method for determining least significant differences (LSD) between treatment means.

RESULTS

SEEDLING PERFORMANCE

ANOVA calculations were completed on the data for shoot heights, shoot fresh weights, and root fresh weights given in Appendices VIII-X. Table II summarizes the total shoot height, shoot weight, and root weight data. The totals are the sum of the data for three seedlings in each pot. The values in Table II are averages of the three replications of each treatment. The differences due to treatments are not significant, but in each case the highest values recorded were for the N4 treatment.

TABLE II

MEAN TOTALS FOR SHOOT HEIGHTS AND WEIGHTS AND ROOT WEIGHTS FOR THE NINE PRECIPITATION TREATMENTS[a]

Treatment	Mean Total Shoot Height, cm	Mean Total Shoot Weight, g	Mean Total Root Weight, g
SN5	103.0	88.2	49.5
S5	104.0	84.3	39.0
N5	97.5	78.8	40.3
CL5	89.2	69.2	38.8
SN4	91.8	92.7	41.8
S4	99.3	74.7	39.5
N4	116.0	106.2	55.8
CL4	100.0	91.5	35.0
CON	105.3	98.2	43.8

[a]Values are the average of three replications.

The Factoral arrangement of this same data is presented in Table III.

TABLE III

FACTORAL ARRANGEMENT FOR MEAN TOTAL SHOOT HEIGHTS AND FRESH WEIGHTS AND ROOT FRESH WEIGHTS[a]

		No Sulfur		Sulfur	
		No Nitrogen	Nitrogen	No Nitrogen	Nitrogen
pH	Data				
5	Height, cm	89.2[c]	97.5[c]	104.0[b,c]	103.0[b,c]
	Weight, g	69.2[c]	78.8[c]	84.3[b,c]	88.2[b,c]
	Root weight, g	38.8[c]	40.3[b,c]	39.0[b,c]	49.5[b,c]
	Treatment	CL5	N5	S5	SN5
4	Height, cm	100.0[c]	116.0[b]	99.3[c]	91.8[c]
	Weight, g	91.5[b,c]	106.2[b]	74.7[c]	92.3[b,c]
	Root weight, g	35.0[c]	55.8[b]	39.5[b,c]	41.8[b,c]
	Treatment	CL4	N4	S4	SN4

[a]Average of three replications.
[b,c]Means for a specific growth parameter with a common superscript are not significantly different at 90% confidence interval.

MYCORRHIZAL DEVELOPMENT

The M-Index means from the data given in Appendix XI are presented in Table IV. An ANOVA F-test showed the treatment differences to be highly significant. The least significant differences were tested at the 5% level; values which do not share a common alphabetical character indicate a significant difference at this level. The two sulfur treatments, S5 and S4, and the N4 treatment had significantly less mycorrhizae than the control and all the remaining treatments, with the exception of SN4. The 2 x 2 x 2 factoral arrangement for the M-Index data is presented in Table V. The analysis of this factoral treatment of the data is summarized in Appendix XI.

TABLE IV

MYCORRHIZAE INDEX AND IAA LEVELS IN ROOTS[a]

Treatment	M-Index	IAA Level, mg IAA/kg dry wt.
SN5	72.3[b]	1.11[c]
S5	28.7[c]	1.10[c]
N5	68.3[b]	16.01[b]
CL5	68.3[b]	0.99[c]
SN4	49.3[bc]	0.93[c]
S4	22.3[c]	0.33[c]
N4	27.3[c]	1.25[c]
CL4	64.0[b]	0.95[c]
CON	72.3[b]	1.05[c]

[a]Average of three replications.
[b,c]Means with a common superscript are not significantly different at 95% confidence interval.

LEVELS OF IAA IN ROOTS

The mean levels of IAA in the roots are also summarized in Table IV (data given in Appendix XII). ANOVA results demonstrate a highly significant difference between treatments, primarily due to the N5 treatment.

NUTRIENT LEVELS

Mean values for the levels of the different nutrients in the seedling needles are assembled in Table VI. The results of the ANOVA calculations demonstrated that there were no significant differences due to treatments for

the nutrients tested (Appendices XIII to XXIII). The levels of N, S, P, K, Ca, and Mg are given in % dry needle weight, while the levels of B, Mn, Zn, Fe, and Al are given as parts per million.

TABLE V

MYCORRHIZAE INDEX REPRESENTD BY 2 X 2 X 2 FACTORAL ARRANGEMENT

		No Sulfur		Sulfur	
		No Nitrogen	Nitrogen	No Nitrogen	Nitrogen
pH	Data				
5	M-Index	68.3	68.3	28.7	72.3
	Treatment	CL5	N5	S5	SN5
4	M-Index	64.0	27.3	22.3	49.3
	Treatment	CL4	N4	S4	SN4

CORRELATION OF DATA

Table VII is a correlation matrix of the major data generated in the experiment. Except for the data involving IAA levels, the values for the correlation coefficients, r, were determined from 27 data points. A significant correlation exists at the 5% level with n-2 degrees of freedom when $|r| > 0.381$. For simple correlations involving the IAA data, r was determined from 25 data points; consequently, significance was determined when $|r| > 0.396$.

TABLE VI

MEAN NUTRIENT LEVELS FOR TREATMENTS

Nutrient Levels, % dry wt.

Treatment	N	S	P	K	Ca	Mg
SN5	1.52	0.15	0.18	0.74	0.29	0.12
S5	1.43	0.14	0.18	0.72	0.25	0.10
N5	1.43	0.17	0.19	0.68	0.29	0.13
CL5	1.52	0.17	0.19	0.80	0.36	0.14
SN4	1.39	0.15	0.18	0.69	0.30	0.11
S4	1.52	0.16	0.20	0.74	0.31	0.12
N4	1.48	0.16	0.19	0.71	0.27	0.12
CL4	1.43	0.16	0.19	0.70	0.30	0.12
CON	1.41	0.15	0.20	0.71	0.28	0.11

Nutrient Levels, ppm

Treatment	B	Mn	Zn	Fe	Al
SN5	27	730	92	49	240
S5	28	630	92	52	177
N5	30	637	97	56	207
CL5	35	760	127	62	263
SN4	32	820	122	56	247
S4	31	727	111	53	230
N4	29	800	98	49	240
CL4	31	757	96	52	253
CON	29	780	79	47	243

TABLE VII

CORRELATION MATRIX

	Variables							
	(1)	(2)	(3)	(4)	(5)	(6)[a]	(7)[a]	(8)[a]
M-Index (1)	-	0.235	0.009	0.005	0.067	0.110	0.138	0.040
IAA Levels (2)		-	0.032	0.120	0.070	0.236	0.119	0.050
Shoot height (3)			-	0.431*	0.366	-0.362	0.147	-0.319
Shoot weight (4)				-	0.583*	-0.436*	0.308	-0.373
Root wt. (5)					-	0.106	0.054	0.004
S Levels[a] (6)						-	0.426*	0.731*
N Levels[a] (7)							-	0.249
P Levels[a] (8)								-

*Denotes significance at the 95% level and 25 d.f.
[a]Nutrient levels in seedling needles.

Two other simple correlations, not easily amenable to a tabular array, were also significant. Presented here, they are:

Levels of IAA/fresh root weight vs. levels of IAA/dry root weight, r = 0.993,

Ca levels in seedling needles vs. Mg levels in seedling needles, r = 0.756. Correlation coefficients between the M-Index and the levels of nutrients in the seedling needles were not significant (Appendix XXIV).

Plots of the change in shoot weight with changing height, change in root weight with changing shoot height, the comparison of shoot fresh weight and levels of sulfur in needles, comparison of sulfur and nitrogen levels in seedling needles, comparison of sulfur levels and phosphorous levels in seedling needles, the IAA levels per fresh root weight and dry root weight, the comparison of calcium levels and magnesium levels in seedling needles, and the M-Index and IAA levels are presented in Fig. 10-17, respectively.

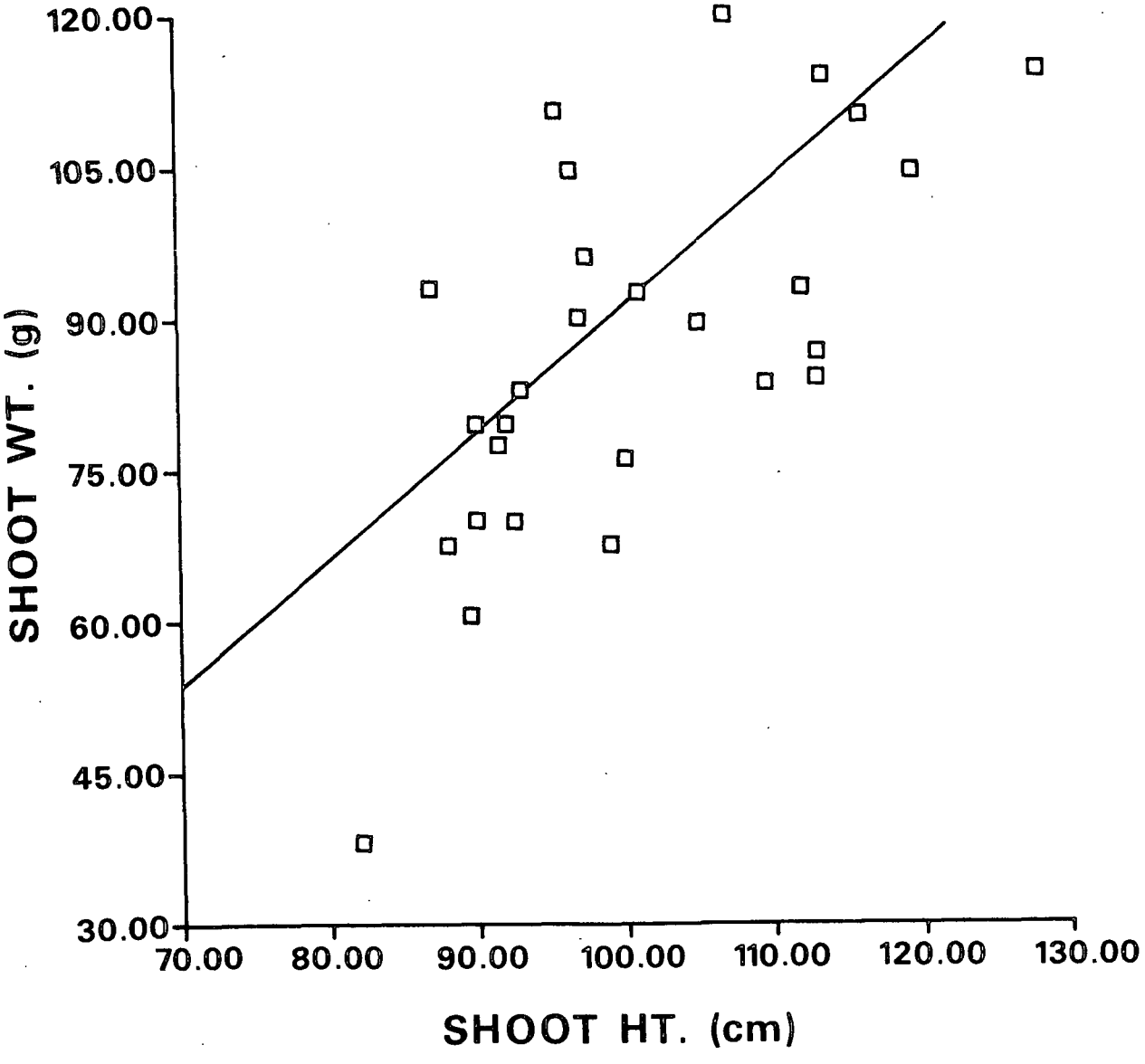

Figure 10. Change in shoot weight with changing height.

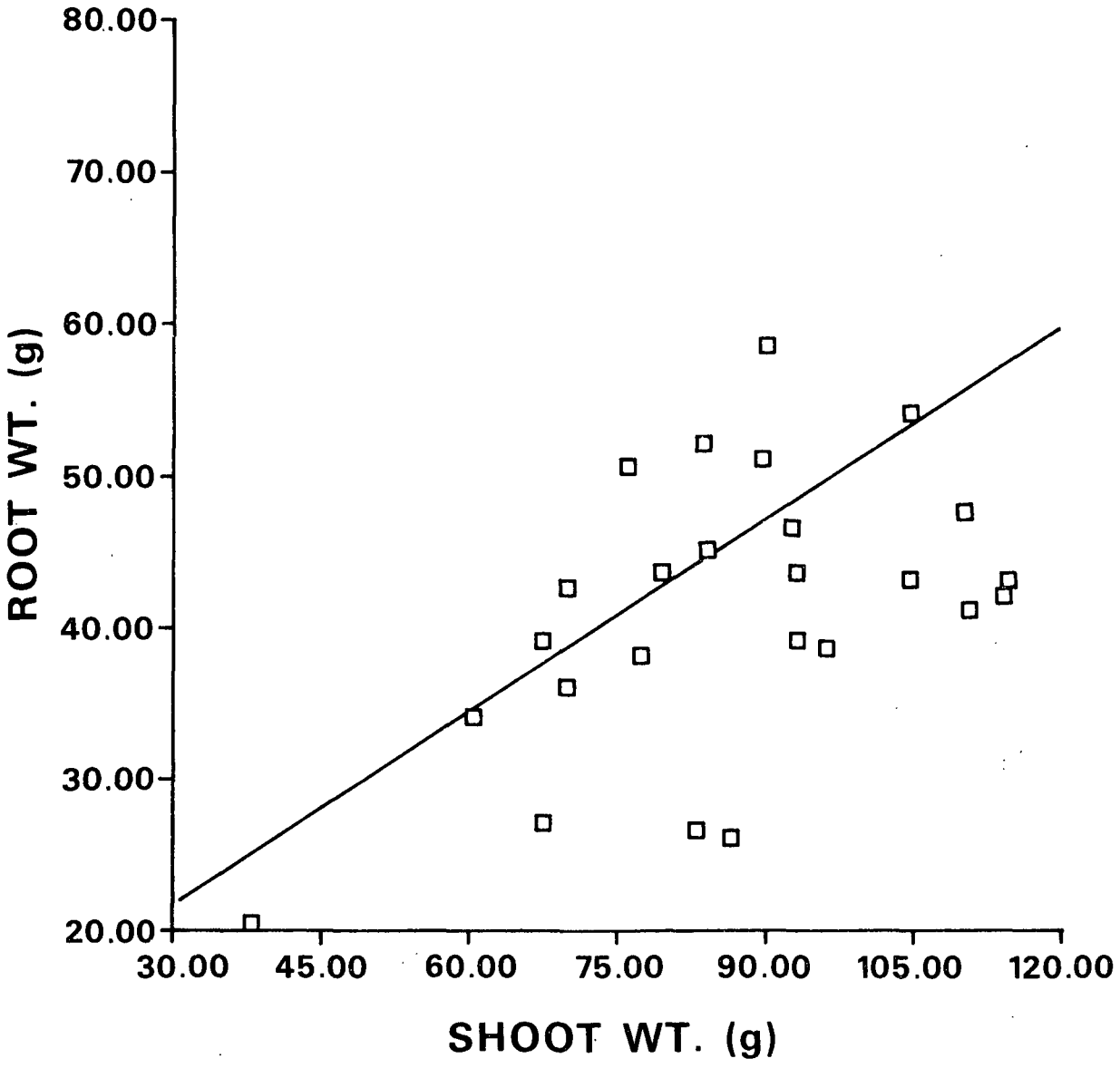

Figure 11. Change in root weight with changing shoot weight.

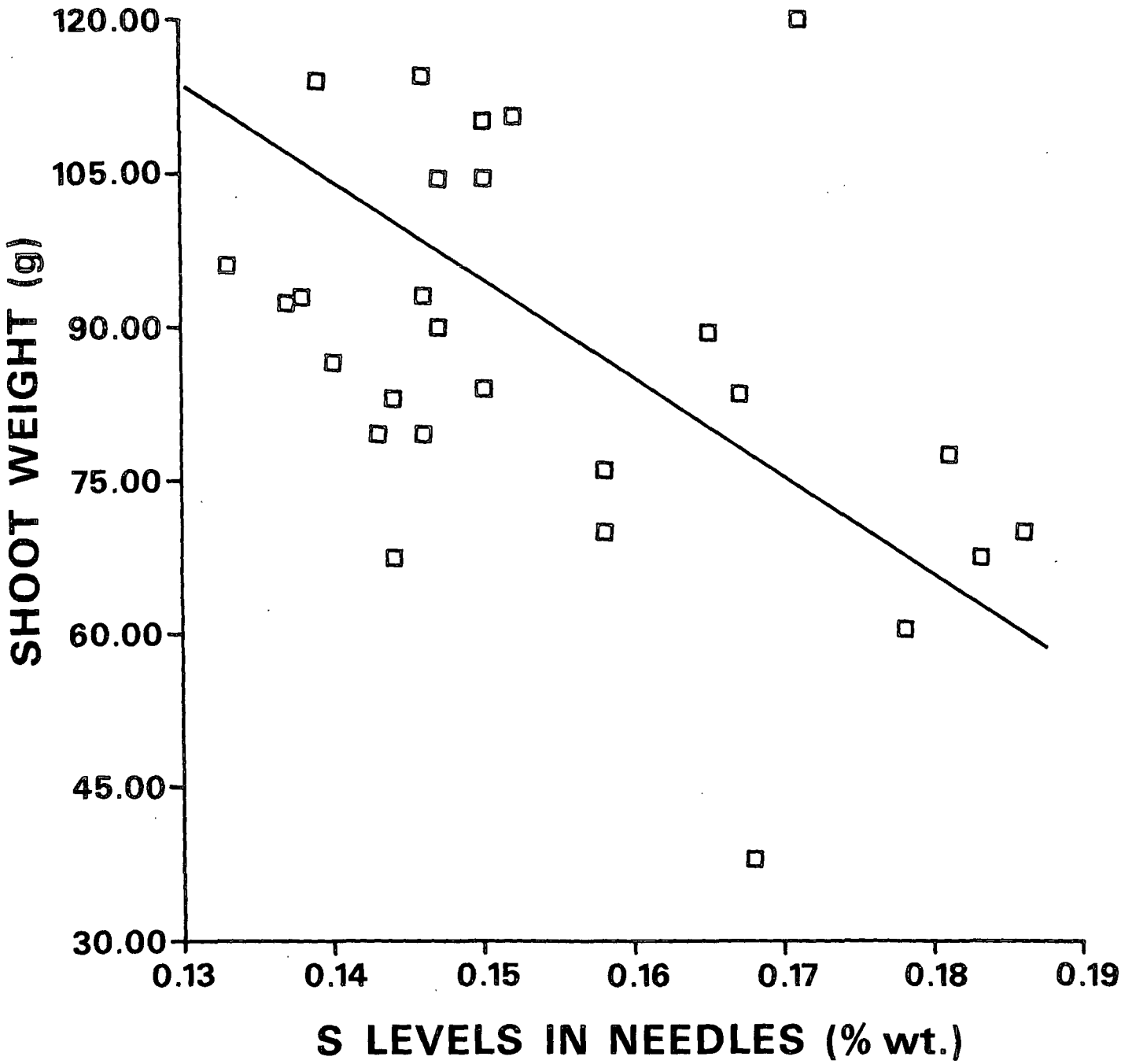

Figure 12. Comparison of shoot fresh weight and levels of sulfur in needles.

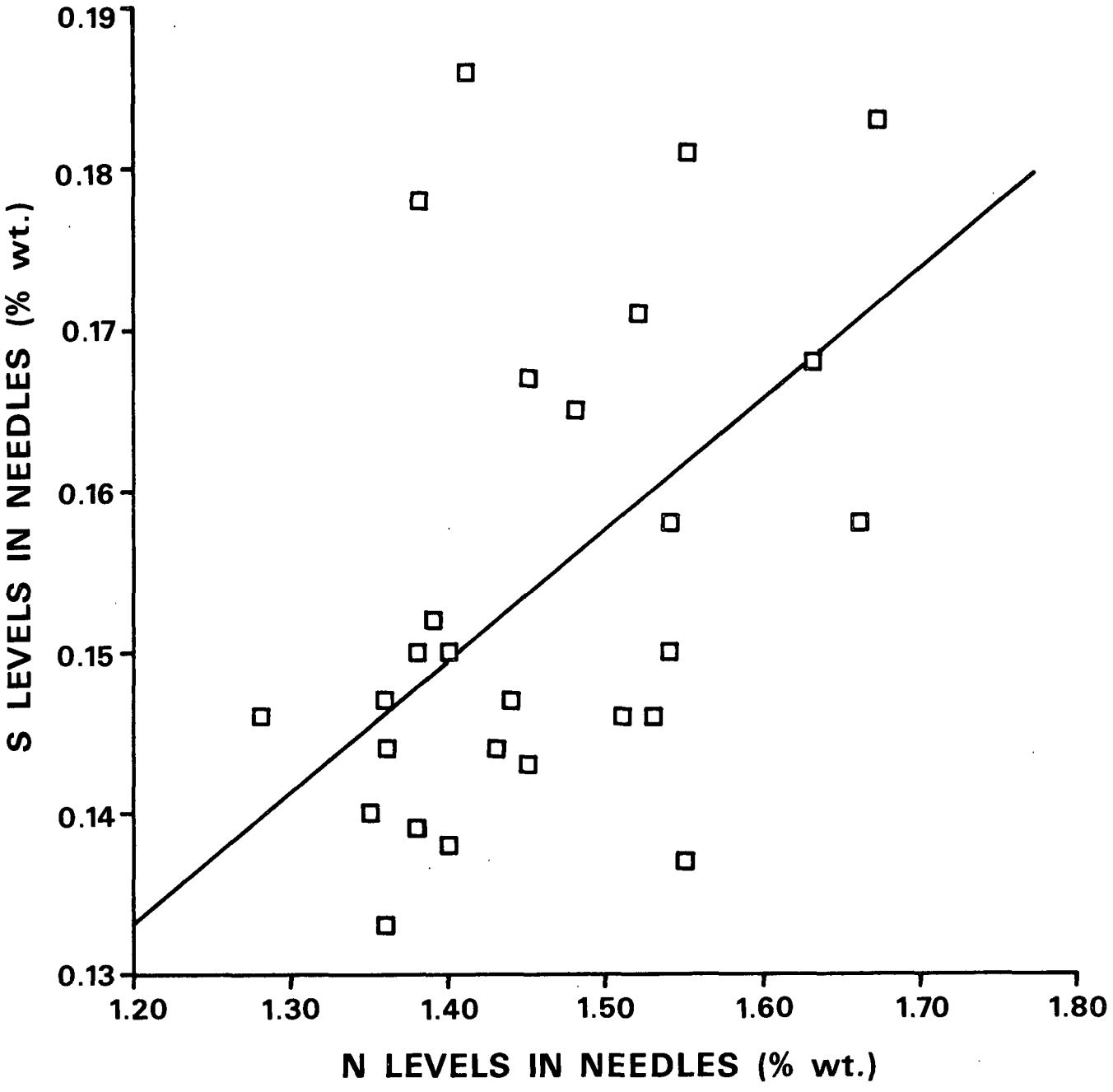

Figure 13. Comparison of sulfur and nitrogen levels in seedling needles.

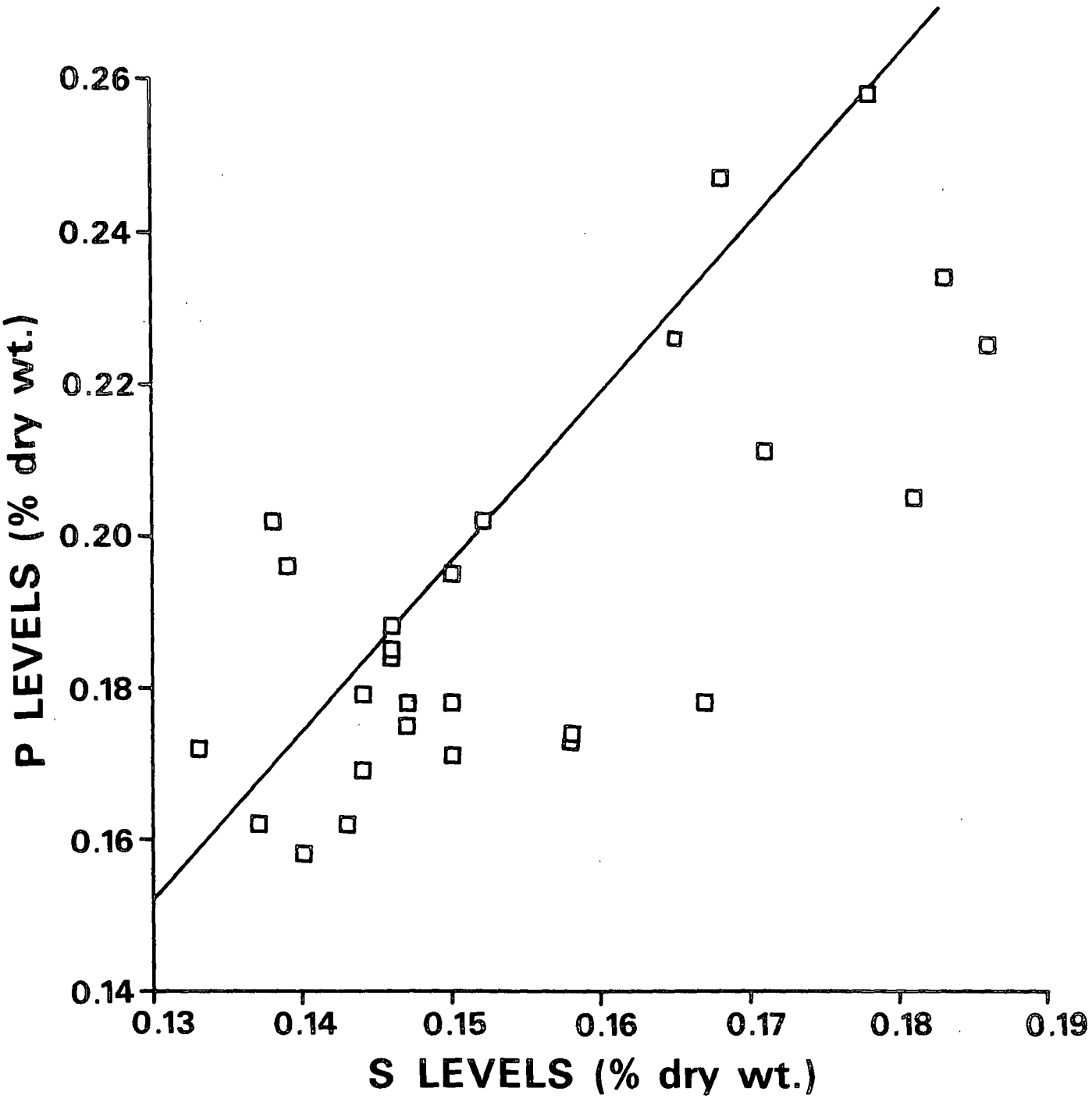

Figure 14. Comparison of sulfur levels and phosphorous levels in seedling needles.

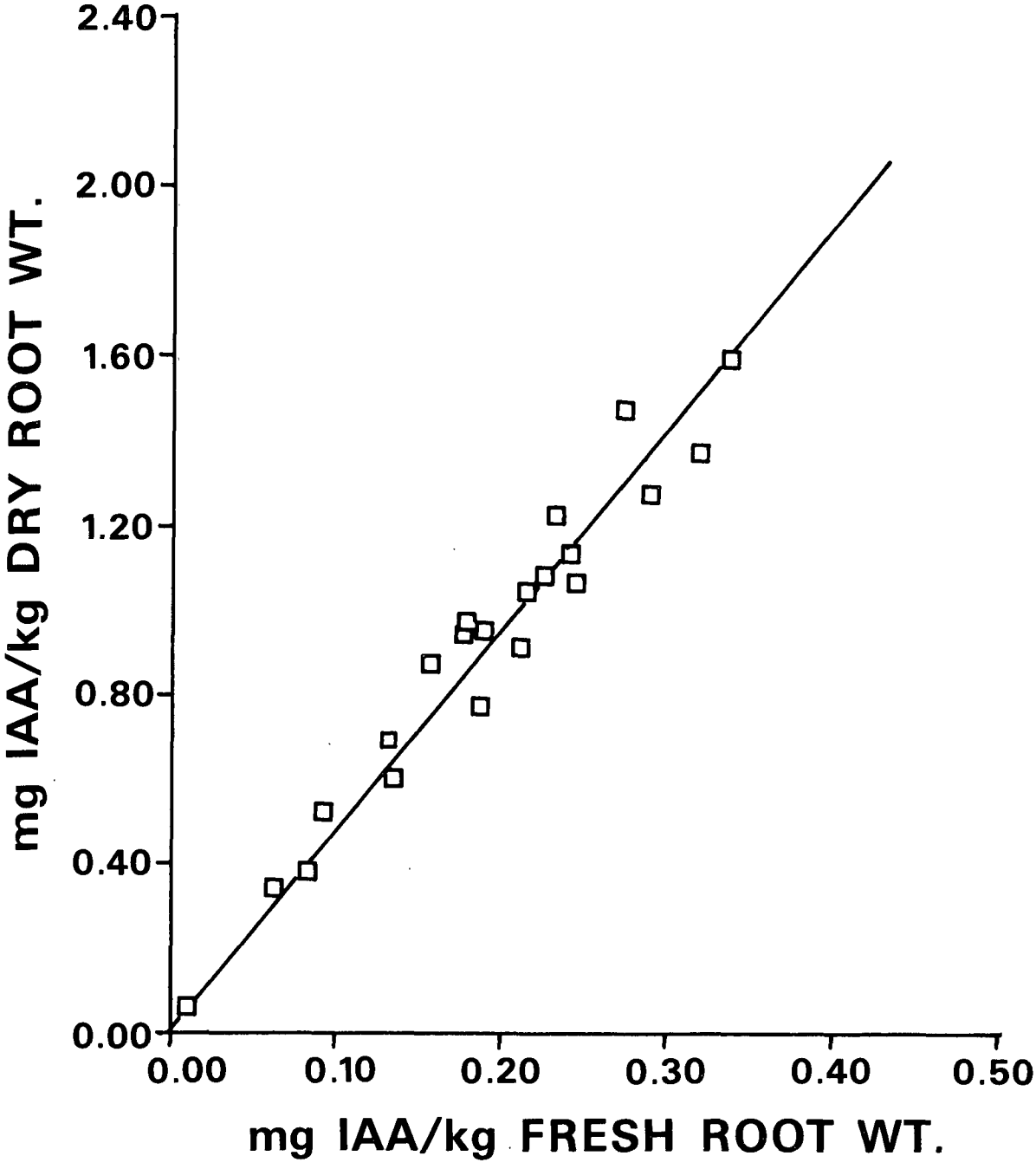

Figure 15. IAA levels per fresh root weight and dry root weight. The values for the N5 treatment did not fit the scale chosen and were excluded from the figure.

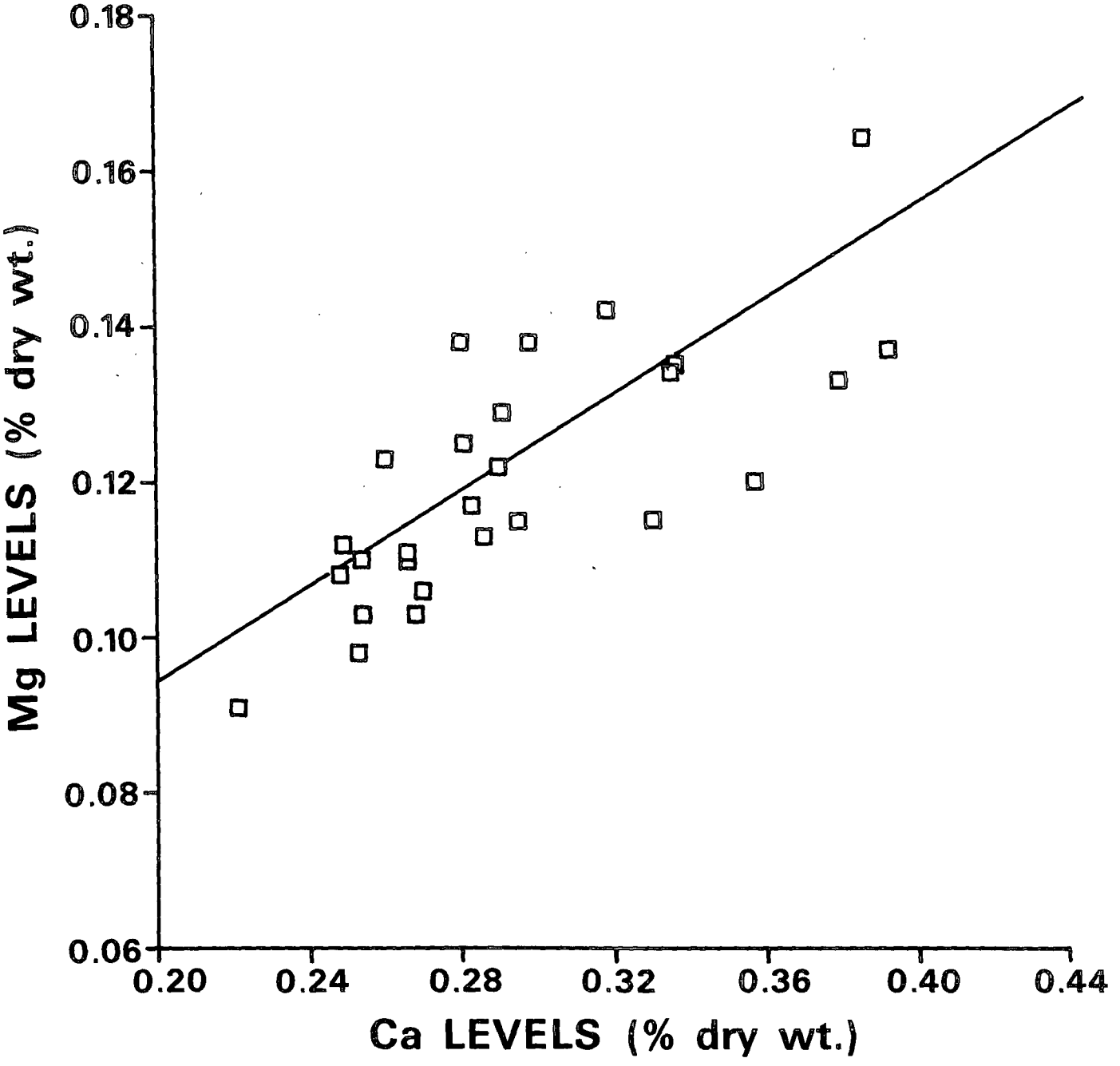

Figure 16. Comparison of calcium levels and magnesium levels in seedling needles.

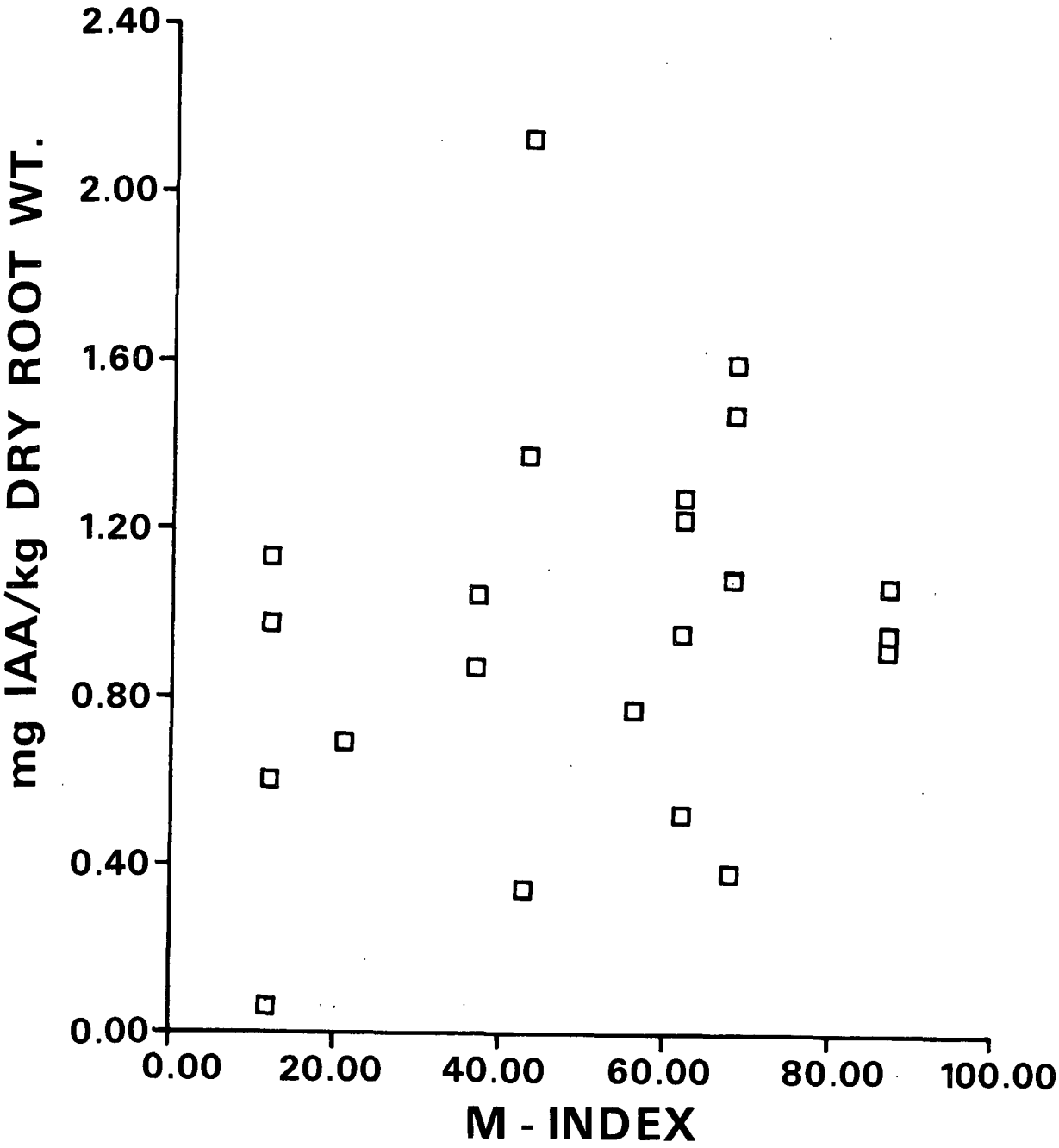

Figure 17. Mycorrhizae index and IAA levels. The values for the N5 treatment did not fit the Y-scale chosen so were excluded from the figure.

DISCUSSION

TREE PERFORMANCE

With the exception of root weight for the SN5 treatment, the control treatment outperformed the acid rain treatments (SN5 and SN4) for shoot height, weight, and root weight (Table II). However, the differences between the control treatments and the acid rain treatments were not great enough to be statistically significant. At this point, it could be argued that the addition of nitrogen, the nutrient most associated with improved tree growth, counteracted any adverse pH effects, but this seems unlikely. The two CL treatments were designed to detect any effects due to changing pH, and these treatments also had no significant influence on tree height, weight, and root weight. This all suggests that acid rain at mild to moderate pH has no adverse effect on tree performance. It is possible that this conclusion is valid only for short term exposures to acid rain; over a longer period of time, the tree's resistance to acid rain could diminish and negative effects could begin to surface.

It is interesting to note the trends that emerge at the 90% level of confidence. At this level, there is a significant difference between the means for the interaction between sulfur and pH (Appendix VIII) on shoot height. Figure 18 illustrates the nature of this interaction. In the absence of sulfur, a decrease in pH resulted in improved shoot height. This improvement was influenced by the N4 treatment, which had a growth response higher than all the other nonsulfur treatments. This result was not surprising, particularly for the difference between the N5 and N4 treatments. Nitrogen is the element most associated with improved tree growth, and in the N5 and N4 treatments, a decrease in pH means an increase in the levels of nitrogen applied. The growth improvement was thus a "fertilizer" effect.

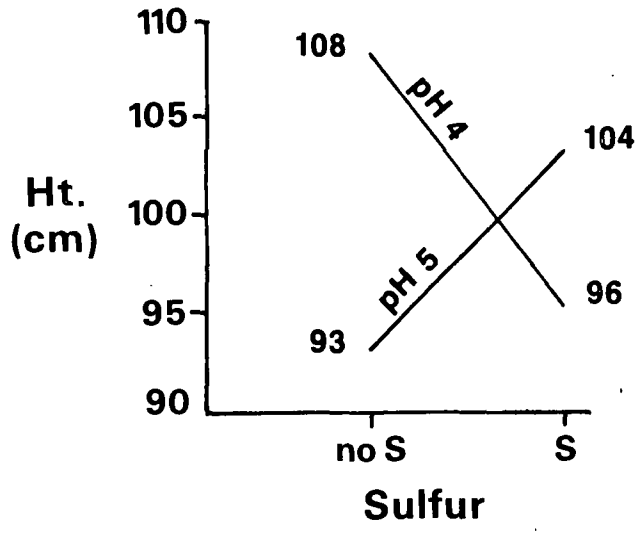

Figure 18. Shoot height as influenced by the sulfur-pH interaction. Differences between means are significant at the 90% confidence level if $|\bar{X}_1 - \bar{X}_2| > 15$. The value 108 represents the mean of the CL4 and N4 treatments; 93, CL5 and N5; 104, S5, and SN5; 96, S4, and SN4.

A similar interaction between sulfur and pH occurred at the 90% confidence level for shoot weight (Appendix IX). Again, in the absence of sulfur, the N4 treatment had a greater weight than the N5 treatment (Table III; differences between means were significant at the 90% confidence level if $|\bar{X}_1 - \bar{X}_2| > 27$) - another fertilizer effect. This time, however, the N4 treatment outperformed the S4 treatment by 42%. Since there was a significant negative correlation between sulfur levels in seedling needles and shoot fresh weight (Fig. 12), there was a negative influence from sulfur with a positive influence from nitrogen. If these influences balance out at the cellular level, then this would explain why the SN treatments did not differ from any of the other treatments.

The fertilizer effect due to nitrogen was again apparent at the 90% confidence level for root weight (Appendix X). Here, too, the N4 treatment showed

the best response, but the differences fitted the statistical model only between N4 and the CL treatments (differences were significant at the 90% confidence level if ≥ 17). This seems logical, since the CL treatments were devoid of any added elements beneficial to root development.

MYCORRHIZAL DEVELOPMENT

ANOVA comparisons demonstrated that there were significant differences in mycorrhizal development due to treatments (Table IV). The LSD test showed the differences due to treatments were due to the S4, S5 and N4 treatments. The expected response of high levels of nitrogen being detrimental to mycorrhizal development confirms earlier studies with mycorrhizae. However, it is interesting to see a similar response for high and low levels of sulfur, as in the S4 and S5 treatments. Apparently, the P. tinctorius mycorrhizae are more sensitive to sulfur than to nitrogen.

Mild acid rain seems to have no adverse effects on mycorrhizal development, as the LSD test demonstrated no significant difference between the SN5 treatment and the control treatment (both of which had well-developed mycorrhizae). The effects of moderate acid rain on mycorrhizal development seem ambiguous, since the LSD test failed to detect a significant difference between the SN4 treatment and all other treatments. Careful consideration of the factoral design (Table V) provides a better understanding of the response to the SN4 treatment.

Although the one-way ANOVA demonstrated a significant difference between the N5 and N4 treatments, the factoral analysis also showed there was a highly significant interaction between nitrogen and sulfur (Appendix XI). Figure 19 shows that in the absence of nitrogen, any addition of sulfur was detrimental to

mycorrhizal development. Also evident from Fig. 19 is the case where mycorrhizal development in sulfur systems was improved when nitrogen was added.

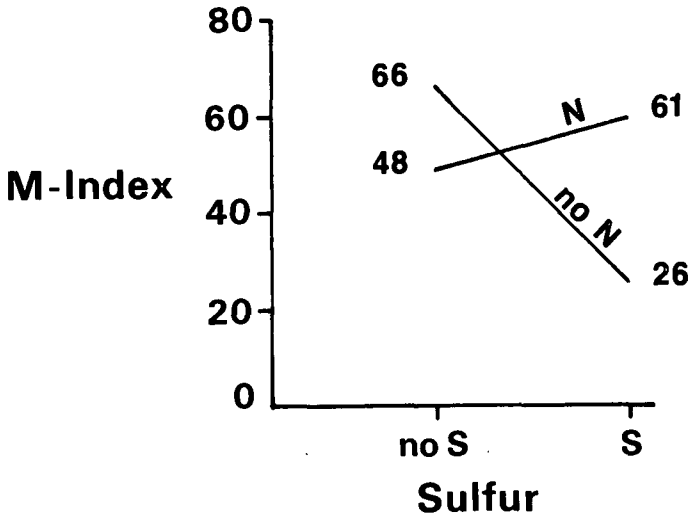

Figure 19. Mycorrhizae index as influenced by the sulfur-nitrogen interaction. Differences between means are significant at the 95% confidence level if $|\overline{X}_1 - \overline{X}_2| > 32$. The value 66 represents the mean of the CL5 and CL4 treatments; 48, N5 and N4; 61, SN5 and SN4; 26, S5 and S4.

Now, examine Fig. 20 which shows the interaction between nitrogen and pH, which is significant at the 90% confidence level (Appendix XI). The difference between the treatment means for the systems with nitrogen was significant at the 95% level between pH 5 and 4. Although this statement mirrors the result between the N5 and N4 treatments in the LSD test for the one-way ANOVA, it also indicts the SN4 treatment, which was not different from any other treatment in the one-way ANOVA.

Accepting the results of the nitrogen-pH interaction and saying that the SN4 treatment is detrimental to mycorrhizal development means risking a Type II error. This risk is greater than the risk associated with accepting the results of the nitrogen-sulfur interaction and committing a Type I error; that is, saying that the SN4 treatment is not detrimental to mycorrhizal development.

This latter conclusion is accepted with one reservation: the mycorrhizae developing in the SN4 treatments were approaching their limit of tolerance to the conditions selected for testing the hypothesis. In other words, a further decrease in pH, or the addition of more SN4 type acid rain, would have adversely affected mycorrhizal development.

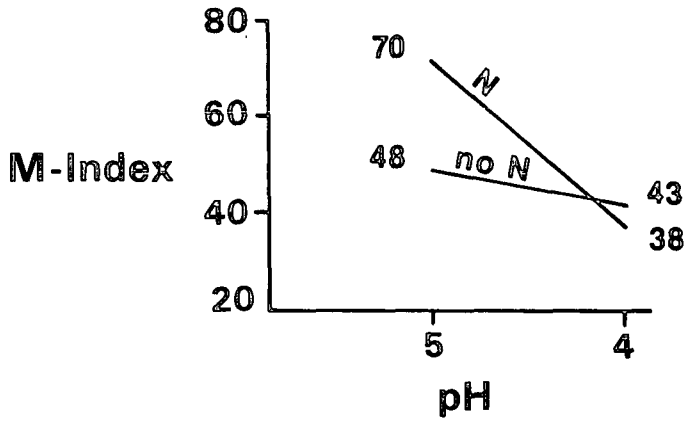

Figure 20. Mycorrhizae index as influenced by the pH-nitrogen interaction. Differences between means are significant at the 95% confidence level if $|\overline{X}_1 - \overline{X}_2| > 32$. The value 70 represents the mean of the N5 and SN5 treatments; 48, CL5 and S5; 43, CL4 and S4; 28, N4 and SN4.

At any rate, the results confirmed part of the previously stated hypothesis. There was sufficient nitrogen in acid rain to interfere with mycorrhizal development, but only in acid rain of the N4 type. Unfortunately, the hypothesis failed to embrace the role of sulfur, which was more adversely influential on mycorrhizal development than nitrogen. However, this was the first indication that sulfur is more detrimental to mycorrhizal development of P. tinctorius on P. taeda L. Previous studies have not examined the role of this element.

Before completing this section, another observation concerning mycorrhizal development is worth noting. As mentioned earlier, the ANOVA results for the CL5 and CL4 treatments showed no significant reduction of mycorrhizae due to

pH. Apart from this, it is no surprise that the CL treatments had M-Indices comparable to the control treatment, since the CL treatments contained none of the ions known to influence mycorrhizal development.

LEVELS OF IAA IN ROOTS

IAA levels varied from 0.33 to 16.01 mg/kg dry weight (Table IV). It is curious that the N5 treatment should have the highest level of IAA and yet demonstrate no corresponding improvement in any of the seedling performance parameters. The N5 treatment did have a large M-Index, but other treatments with similar high M-Indices did not have the same high levels of IAA; consequently, the correlation coefficient between IAA levels and M-Index was not significant (Fig. 17).

Since the hypothesis stated that critical levels of nitrogen would disrupt IAA synthesis and cause mycorrhizae to disappear, high levels of IAA were expected for the seedlings with high M-Indices, and low levels of IAA were expected for the seedlings with low M-Indices. Even though the results ran contrary to these expectations, there are at least two possible explanations for this lack of correlation (more on this in the next section).

One possible explanation for the unusual response from the N5 treatment is that, at the time of harvesting, the N5 seedlings were in an elevated metabolic state. That is to say, the levels of IAA were cyclical for all the seedlings, but the N5 seedlings just happened to be harvested at a point in time when the cycle was near a peak. Whether this increase in IAA production was due to a *de novo* synthesis or a release from bound forms of IAA (or both) is unknown.

THE ROLE OF IAA IN MYCORRHIZAE FORMATION

The lack of a significant correlation between the levels of IAA in the roots and the extent of mycorrhization, as characterized by the M-index, merits special consideration. It is not unreasonable to question the validity of the method for determining IAA levels or the M-index as two possible sources for this unexpected result.

Shortly after the development of the method for determining IAA levels in roots, concern arose over whether long term storage of roots at -20°C would have any effect on the IAA. A large sample of roots was collected, macerated, mixed, and then divided into six equal portions. The samples were analyzed at weekly intervals and the results displayed a good agreement among the values (Appendix XXVI). Not only did this demonstrate that long term cold storage of roots did not influence the levels of IAA, but it also showed that the analysis, when repeated, was reliable.

The determination of the M-index is certainly more susceptible to error, since a subjective response is necessary for its evaluation. However, there can be no mistaking roots which show extreme abundances of mycorrhizae with those which display very little or no mycorrhizae. A comparison of the mean values of IAA in roots from these two categories shows no significant difference in the IAA levels (Appendix XXVII). Clearly, then, some other explanation is in order to explain the lack of correlation between the levels of IAA and the degree of mycorrhization.

One possible explanation for this lack of correlation may be that levels of IAA in mycorrhizal roots fluctuate. At any given time, the value detected may be a maximum, a minimum, or something in between. Work performed in connection

with this thesis showed that levels of IAA in cultured wild carrot tissues go from a maximum to a mimimum and then again to a maximum over a period of 28 days (Appendix XXVIII). If mycorrhizal roots are also subject to varying levels of IAA, then the result of the N5 treatment would be no mystery: it could be that the N5 seedlings just happened to be harvested at a time when the IAA levels had fluctuated to a maximum.

Another possible explanation for this lack of correlation may be that not all mycorrhizal infections are manifested externally. Heazel (34) has shown that roots classified as nonmycorrhizal based on physical characteristics visible to the naked eye did indeed turn out to be mycorrhizal upon cross-sectional examination at 100X. Concerning the present study, if the roots with low M-indices still had extensive internal mycorrhization, then the mycorrhizae could still be influencing the levels of IAA in the roots. While this may very well be the explanation for the lack of correlation between the IAA levels and the M-index, it raises another important question: Why are levels of IAA in one mycorrhizal root producing pronounced external structural changes while similar levels in another mycorrhizal root are not?

At this point, it is necessary to reconsider the literature on IAA and mycorrhizal development. Although it is documented that IAA produces the swelling and forking characteristics of mycorrhizal roots, it is important to realize that such documentation is causal rather than analytical, and is independent of any consideration of the molecular action of IAA within the system. This leads to a second possible explanation for the lack of correlation between IAA levels and the M-index: mycorrhizal structure is determined by conjugates of IAA and not free IAA.

Conjugates of IAA are known to exist as esters with glucose and other simple sugars or as amides with amino acids and peptides (35,36,37). Liberation of IAA from esters requires treatment with 1N NaOH, while hydrolysis of peptidic IAA requires even harsher conditions (36), neither of which were applied in the extraction procedure. What follows, then, is that the IAA responsible for the formation of mycorrhizal structure escaped detection because it was chemically bound to other biochemicals. This suggestion is not novel: Slankis (19) made a similar proposal to explain an unexpected absence of IAA in pine root extracts subjected to paper chromatography.

While the purpose of this thesis was to gauge only the level of free IAA and its relation to mycorrhizal structure, the question raised about the role of bound IAA was interesting enough to investigate on a preliminary basis (Appendix XXIX). The results of this test showed that conjugates of IAA do exist in mycorrhizal roots. Although no evidence of peptidic IAA appeared in this test, ester bound IAA was detected at a level of 14% of the total IAA detected.

CONCLUSIONS

Soil applications of mild acid rain, as characterized by a solution of sulfuric and nitric acid at a 7:3 ratio and adjusted to pH 5, had no effect on the development of Pisolithus tinctorius on Pinus taeda L. to form mycorrhizae. The treatment with moderate acid rain (i.e., acid rain at pH 4) approached the limit of tolerance at which this mycorrhizal association could develop normally. The individual components of acid rain at pH 4 were more detrimental to mycorrhizal development acting alone than acting together, and the development of P. tinctorius on P. taeda L. was affected more adversely by sulfur than nitrogen. A change in hydrogen ion concentration (i.e., decreasing pH from 5.6 to 5.0 to 4.0 with hydrochloric acid) did not influence this mycorrhizal association.

The nitrogen component of acid rain produced a fertilizer effect which improved tree growth at the 90% level of confidence. A detrimental influence by the sulfur component counteracted the gains made in shoot weight by nitrogen addition. There was no improvement or decline in tree performance due to acid rain over a period of nine months.

High and low levels of free indole acetic acid in the P. taeda L. root systems inoculated with the mycorrhizal fungus P. tinctorius did not correlate with high and low degrees of mycorrhizal formation. IAA levels in mycorrhizae roots may either fluctuate over time or are dependent on both free and bound forms of IAA and as a result were not correlated mycorrhizal development.

A suitable analytical method for determining levels of free IAA in mycorrhizal roots was established. The method is adaptable to measuring IAA levels in tissue culture systems, as well as levels of ester and amide bound IAA in tree roots.

FUTURE WORK

The role of bound IAA in the formation of mycorrhizal roots is an interesting prospect for elucidating the process by which mycorrhizal characteristics are determined. The Bandurski and Schulze method (31) for determining levels of bound IAA in tissue extracts is easily adaptable to the IAA determination method developed in this thesis. It is evident from preliminary tests, however, that the procedure should be slightly modified to improve the purification of the peptide hydrolysis sample.

Now that it is known that the components of acid rain do not work independently of one another on mycorrhizae, definite tolerance levels should be determined for the symbiont. This would mean ignoring the effects of the isolated ions and concentrating instead on various ionic ratios at pH's of 4 and less. It would be desirable to conduct such an experiment over a longer period of time to see if levels of these ions accumulate to the point where the interaction effect is diminished and the action of the individual ions takes over. This could possibly be determined in a tissue culture system.

The fertilizing effect of acid rain may be more pronounced over a longer period of time or at lower pH's, but care should be taken before ascribing this action as a "benefit." The effects could be short lived, and the action of acidic precipitation over a period of a few years could outweigh the short term growth improvements. This points to the need for lengthier and more detailed studies about the influence of acid rain on tree growth. Other encompassing points include the effects on tree foliage and the mobilization of soluble nutrients in the soil. The effects of acid rain might be quite different on soils of high cation exchange capacity and high levels of base saturation (pH 6 to 7).

GLOSSARY AND SPECIAL ABBREVIATIONS

base peak	the peak in a mass spectrum produced by the most abundant molecular fragment
bifurcated	a root tip divided into two branches
estimator	a statistically generated value used to replace a missing data point
Hartig net	a network of fungal hyphae extending into the first few layers of a root cortex
lateral	a root branching out from the side of another root
mantle	a mass of fungal hyphae surrounding the exterior of a root tip
molecular ion	the peak in a mass spectrum equivalent to the molecular weight of the compound under scrutiny
outlier	a data point statistically determined to be anomalous
retention time	the interval in gas chromatography between injection of a compound and its appearance as an electrical signal from the detector
ANOVA	Analysis of variance
CL5	Acid rain composed of hydrochloric acid at pH 5
CL4	Acid rain composed of hydrochloric acid at pH 4
CON	Control group representing normal rainfall at pH 5.6
d.f.	Degrees of freedom
HFBI	Heptafluorobutyrylimidazole, a derivatizing reagent
IAA	Indole acetic acid, a plant growth hormone (natural auxin)
LSD	Least significant difference, a statistical test used to identify individual treatment(s) which give significant F-test results
M-Index	Mycorrhizae Index
N5	Acid rain composed of nitric acid at pH 5
N4	Acid rain composed of nitric acid at pH 4

$1\text{-}^{14}C$-IAA	Radioactive indole acetic acid with C-1 being the heavy isotope
P	Probability
r	Correlation coefficient
R_f	Ratio between the distance a compound traveled and the distance the solvent traveled in thin layer chromatography
S5	Acid rain composed of sulfuric acid at pH 5
S4	Acid rain composed of sulfuric acid at pH 4
SIM	Selected ion monitoring, a mode in mass spectrometry where all but a few chosen ions are eliminated from the mass spectrum; it allows an increase in machine sensitivity
SN5	Acid rain composed of sulfuric and nitric acid at pH 5
SN4	Acid rain composed of sulfuric and nitric acid at pH 4
TLC	Thin layer chromatography

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APPENDIX I

MODIFIED MELIN-NORKRANS MEDIUM

$CaCl_2$	0.05	g
NaCl	0.025	g
KH_2PO_4	0.5	g
$(NH_4)_2HPO_4$	0.25	g
$MgSO_4 \cdot 7H_2O$	0.15	g
$FeCL_3$ (1%)	1.2	mL
Thiamine HCl	3	g
Glucose	10	g
H_2O	1	L

No pH adjustment is made.

APPENDIX II

MYCORRHIZAE INDEX

The Mycorrhizae Index was designed to give an indication of the relative degree of infection among the root systems of the experimental seedlings. Determination of the M-Index value starts by assigning the root system into one of the four categories listed below:

Rank	Qualitative Description	% Ectomycorrhizal Short Roots
++++	Excellent	greater than 75%
+++	Good	50-74%
++	Moderate	25-49%
+	Poor	24% or less

For example, suppose the initial reaction to some freshly exhumed seedling roots was that it had "good" mycorrhizal formation. Three random root sections would then be chosen and the number of mycorrhizal roots per ten laterals (or short roots) would be counted. In this hypothetical root system, 5, 6 and 8 were the numbers on mycorrhizal roots in the selected sections, so the average is 63%. Since this agrees with the range of % Ectomycorrhizal Short Roots corresponding to good, three pluses are recorded in the notebook and the procedure is then applied to the next sample.

Suppose, instead, that the initial reaction to the previous example had been "excellent" instead of "good." The three pluses would still be recorded in the notebook, but the count would be repeated on three more different random root sections. If the average of the second count again fell into the 50-74% range, the first recording of the three pluses would be deemed sufficient. If the second count fell into the greater than 75% range, then, in addition to the

three pluses already recorded, four pluses would be written down. In some instances, comments were recorded along with the rank to aid in the description of the roots.

The final process in determining the M-Index involves converting the rank into a numerical value so that the results would be suitable for statistical evaluation. Merely assigning the values 1, 2, 3 or 4 (or 1.5, 2.5, 3.5 where two ranks were recorded) to the ranks was not an adequate procedure, since ANOVA results of these values showed significant differences between the treatments, but the least significant differences method failed to single out the different treatments due to the fact that many of the means had the same value. To eliminate this problem, diversity was introduced into the treatment values through the following modifications: (1) Assign to single entry pluses the mean of the range to which they correspond; (2) Assign to dual entry pluses the mean between the value of the first plus entry and the upper (or lower) limit of the range in the direction of the second plus entry. The schematic below gives a clearer description of this procedure.

SCHEMATIC SHOWING M-INDEX DETERMINATION

		++		+++		++++		
DUAL VALUE ENTRIES		(18)		(43)		(68)		LOWER TO HIGHER
	+		++		+++		++++	
SINGLE ENTRIES	(12)		(37)		(62)		(87)	
		+		++		+++		
DUAL VALUE ENTRIES		(31)		(56)		(81)		HIGHER TO LOWER

Tabulated below are the ranks assigned to the mycorrhizal roots examined on the day of harvesting and the M-Index value determined on the basis of the ranks.

Treatment	Block	Rank	M-Index	Comments
SN5	1	+++ to ++++	68	Mantle present
	2	+++	62	Mantle appeared dried out
	3	++++	87	Much mantle and clusters
S5	1	+	12	No mantle
	2	+++	62	Mantle present
	3	+	12	No mantle
N5	1	+++	62	Mantle present
	2	+++	62	Slight mantle formation
	3	++++ to +++	81	Lots of mantle
CL5	1	++++	87	Very abundant mycorrhizae
	2	+++ to ++	56	Mantle present
	3	+++	62	Mantle present
SN4	1	++	37	No mantle
	2	+++ to ++++	68	Lots of clusters
	3	++ to +++	43	No mantle
S4	1	++ to +++	43	No mantle
	2	+	12	No mantle
	3	+	12	No mantle
N4	1	++ to +	31	Very little mantle
	2	+ to ++	18	No mantle
	3	++ to +++	43	Mantle present
CL4	1	++++	87	Mantle present
	2	++	37	No mantle
	3	+++ to ++++	68	Mantle present
CON	1	+++ to ++++	68	No mantle, many clusters
	2	+++	62	Mantle present
	3	++++	87	Very abundant mycorrhizae

APPENDIX III

PLANT ANALYSIS

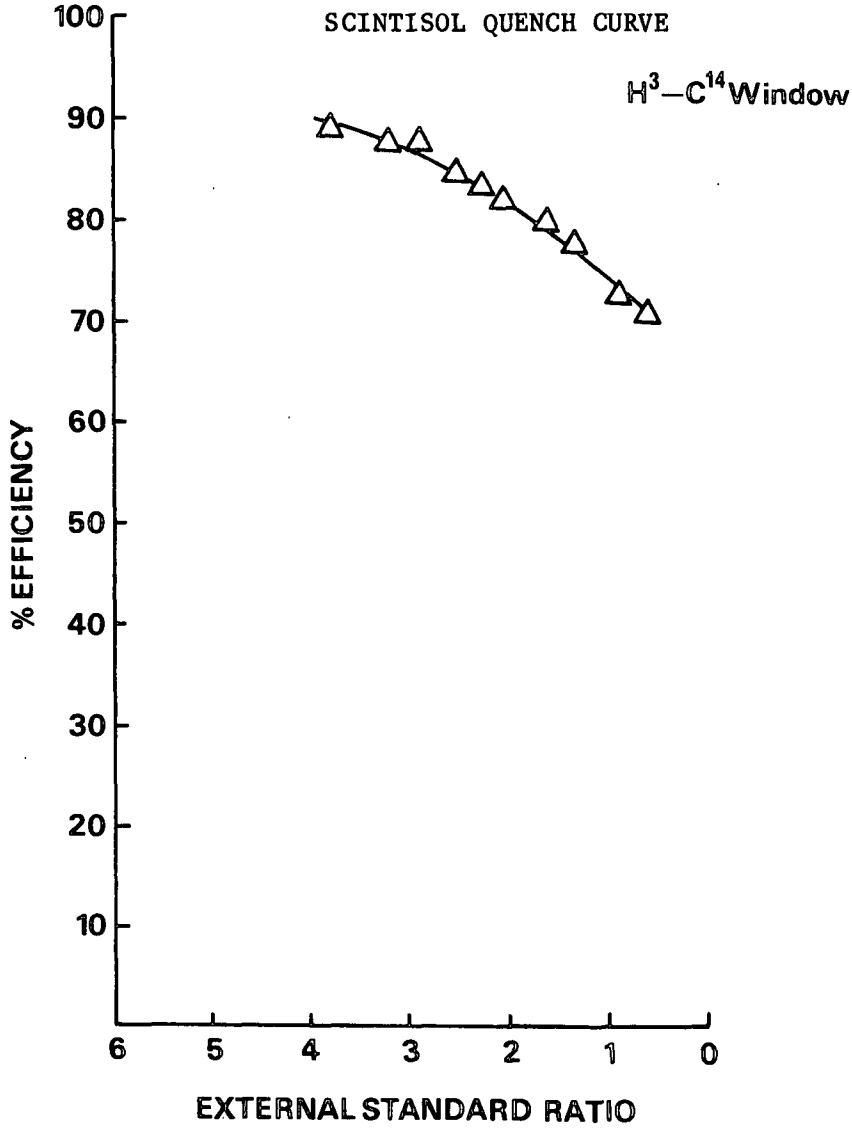
Elemental analysis on plant samples at the University of Wisconsin-Extension Soil & Plant Analysis Lab (806 South Park Street, Madison, WI 53715) are routinely performed for N, P, K, Ca, Mg, S, B, Mn, Zn, Cu, Fe, Al and Na. All but N are analyzed simultaneously by a plasma emission spectrophotometer (Applied Research Labs, Model 34000; inductively-coupled Plasma Emission Quantometer coupled with a Digital Equipment Corporation PDP 11/34 Computer). Nitrogen is determined on a separate sample via a semimicro Kjeldahl procedure.

For the elemental analysis by the plasma emission spectrophotometer, 0.500 g of the dried sample is weighed out and placed in a Folin digestion tube. Sample digestion is first performed with 5 mL of 15N nitric acid for 40 min at 100°C. Then 3 mL of 1N perchloric acid is added, and the second digestion step continues for 90 min at 215°C. Afterward, the sample is refluxed and then diluted to 50 mL. When the sample has cooled, it is then run on the plasma emission spec. Calibration curves are constructed from stock solutions of the various elements purchased from lab supply firms.

APPENDIX IV

QUENCH CURVE FOR SCINTISOL SCINTILLATION COCKTAIL

Monroe (30) constructed a Scintisol scintillation cocktail quench curve by using a known amount of a standard ^{14}C compound, ^{14}C-N-hexadecane. The external standard was run and plotted against the % efficiency of the counting. The sample was quenched sequentially with $FeCl_3$, yielding the following curve for the Scintisol cocktail. The curve was used to determine the efficiency of counting of the 1-^{14}C-IAA internal standard.



APPENDIX V

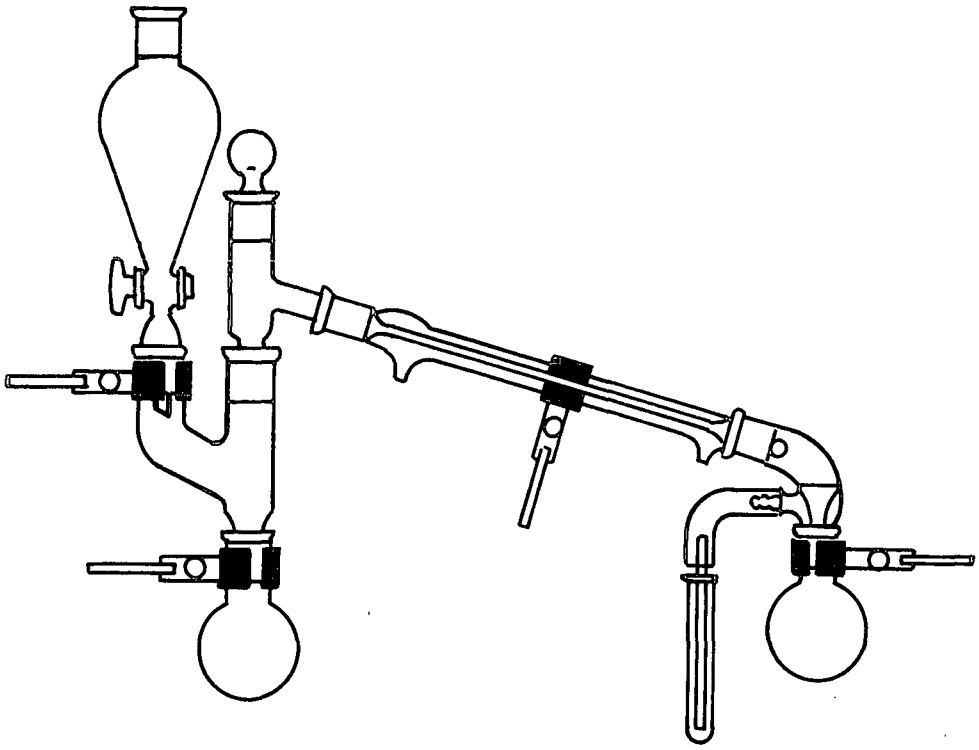
SYNTHESIS OF DIAZOMETHANE

Methylation of IAA to form the first derivative, methyl 3-indolylacetate, requires diazomethane, a toxic compound which has been reported to explode spontaneously upon contact with ground-glass joints. Consequently, elaborate care must be taken in its preparation and use. All work must be performed behind a safety shield and underneath a well-ventilated hood.

A special diazomethane generator was purchased (Aldrich Chemical Company, Inc., Milwaukee, WI) which has no ground glass joints (see illustration on the following page). Ethanol (95%, 12.5 mL) is added to a solution of KOH (2.5 g) in water (4 mL) in a 50 mL distilling flask equipped with a dropping funnel and a condenser set downward for distillation. The condenser is connected to two receiving flasks in series, the second of which contains 10-15 mL of ether. The inlet tube of the second receiving flask dips below the surface of the ether. Both flasks are cooled to 0°C.

The flask containing the alkali solution is heated in a water bath to 65°C, and a solution of 10.75 g of Diazald (n-methyl-N-nitroso-p-toluenesulfonamide) in about 100 mL of ether is added through the dropping funnel. The rate of addition is adjusted to equal the rate of distillation. When the dropping funnel is empty, an additional 20 mL of ether is added until the color of the distilling ether is clear. The two ethereal distillates are combined, and approximately 1.5 g of diazomethane are synthesized.

DIAZOMETHANE GENERATOR



APPENDIX VI

CALCULATION OF IAA LEVELS IN ROOTS

The following lists the stepwise procedure for calculating the levels of IAA in the root samples. Values used in the calculation are taken from a representative sample (the roots in Block 2 of the CL5 treatment).

I. Loss of Internal Standard During Extraction

A. Pre-extraction Level

No. scintillation counts	=	3		
Sample size	=	1 mL		
Counts (dpm/mL)	=	2054.26	2020.64	1986.34
Quench factor	=	2.41	2.32	2.39
Efficiency (from curve)	=	83%	83%	83%
Adjusted counts (dpm/mL)	=	2468.17	2448.78	2390.30
Average	=	2435.75 dpm/mL		
Conversion factor (E)	=	(59 mCi/mmol x 2.2 x 10^9 dpm/mCi)$^{-1}$		
Volume "spiked" CH_3OH	=	117 mL		

AMT. 1-^{14}C-IAA ADDED TO ROOTS = 2435.75 dpm/mL x 117 mL x E

= 2.20 x 10^{-6} mmol

B. Post-extraction Level

No. scintillation counts	=	3		
Sample size	=	0.1 mL		
Counts (dpm/0.1 mL)	=	5815.67	5841.16	5765.41
Quench factor	=	4.71	4.80	4.95
Efficiency	=	97%	98%	99%
Adjusted counts (dpm/0.1 mL)	=	5982.58	5975.00	5843.12
Average	=	5933.57 dpm/mL		

AMT. 1-^{14}C-IAA RECOVERED = 5933.57 dpm/0.1 mL x 1 mL x E

= 4.57 x 10^{-7} mmol

C. Extraction Efficiency

PERCENT RECOVERY = $(4.57 \times 10^{-7}/2.20 \times 10^{-6}) \times 100$

= 20.8%

II. Level of IAA in Root Sample

A. Fresh and Dry Root Weights

Root fresh weight = 39.0 g

Root dry weight (gross) = 10.21 g

Root dry weight (tare) = 0.82 g

Root dry weight = 9.39 g

B. Calibration Curve

FRN*	Amt. IAA Injected (g)	385 Peak Area
8358	2.0×10^{-8}	21477
8359	1.2×10^{-8}	13761
8360	8.0×10^{-9}	11440
8361	4.0×10^{-9}	6071
8362	2.0×10^{-9}	4084

Plot amount IAA vs. peak area; use least squares analysis on TI-55-II hand calculator to obtain slope and intercept.

Slope = 9.560×10^{11} g^{-1}

Intercept = 2572 (dimensionless)

AMT. IAA = (peak area - 2572)/9.560×10^{11} g^{-1}

*File reference number.

APPENDIX XXII

IRON LEVELS IN SEEDLING NEEDLES, ppm

DATA

pH	Treatment				
	SN	S	N	CL	CON[a]
5	55	45	53	65	45
	50	62	60	52	47
	43	48	56	69	49
4	62	46	47	45	--
	48	59	50	50	--
	58	55	50	61	--

[a]Control values are at pH = 5.6.

ONE-WAY ANOVA

Source of Variation	SS	DF	MS	F	P
Blocks	37.852	2			
Treatments	508.296	8	63.537	1.406	0.266
Error	722.815	16	45.176		
Total	1268.963	26			

APPENDIX XXI

ZINC LEVELS IN SEEDLING NEEDLES, ppm

DATA

pH	Treatment				
	SN	S	N	CL	CON[a]
5	118	80	76	102	70
	72	67	75	124	80
	84	126	137	165	88
4	142	82	96	115	--
	66	149	89	80	--
	157	101	109	98	--

[a]Control values are at pH = 5.6.

ONE-WAY ANOVA

Source of Variation	SS	DF	MS	F	P
Blocks	4351.185	2			
Treatments	5591.185	8	698.898	0.932	>0.500
Error	11984.148	16	749.009		
Total	21926.518	26			

Portion	Level IAA Detected, g
1	1.03×10^{-8}
2	1.16×10^{-8}
3	2.50×10^{-8}
Total	4.69×10^{-8}

Assuming Portion 1 represents the base line for free IAA, and the difference between Portion 2 and 1 the base line for ester-bound IAA, then the contributions break down into the following:

	1	2	3
Free	1.03	1.03	1.03
Ester		0.13	0.13
Amide			1.34

These values represent a lower limit of 6% for the contribution of ester-bound IAA to total IAA, and 29% for amide bound.

APPENDIX XII

LEVELS OF IAA IN ROOTS, mg IAA/kg dry weight

DATA

pH	Treatment				
	SN	S	N	CL	CON[a]
5	1.47	0.97	16.57	0.95	1.59
	0.95	1.22	15.24	0.77	0.52
	0.91	1.13	16.24[b]	1.27	1.06
4	1.04	0.34	0.69	0.92	--
	0.38	0.06	0.94[b]	0.87	--
	1.37	0.60	2.12	1.08	--

[a]Control values are at pH = 5.6.

ONE-WAY ANOVA

Source of Variation	SS	DF	MS	F	P
Blocks	1.398	2			
Treatments	605.536	8	75.692	453.245	0.001
Error	2.334	14[c]	0.167		
Total	609.268	24			

[b]Estimators replacing outliers at 95% confidence interval.
[c]Reflects loss of 2 degrees of freedom due to estimators.

APPENDIX XI

MYCORRHIZAE INDEX VALUES

DATA

pH	Treatment				
	SN	S	N	CL	CON[a]
5	68	12	62	87	68
	62	62	62	56	62
	87	12	81	62	87
4	37	43	21	87	--
	68	12	18	37	--
	43	12	43	68	--

[a]Control values are at pH = 5.6.

ONE-WAY ANOVA

Source of Variation	SS	DF	MS	F	P
Blocks	198.222	2			
Treatments	10625.333	8	1328.167	3.690	0.012
Error	5759.111	16	359.944		
Total	16582.667	26			

TWO X TWO X TWO FACTORAL ARRANGEMENT

Source of Variation	SS	DF	MS	F	P
Sulfur	1148.167	1	1148.167	3.271	0.086
Nitrogen	433.500	1	433.500	1.235	0.282
Sulfur-nitrogen	4320.167	1	4320.167	12.307	0.003
pH	2090.661	1	2090.661	5.956	0.025
Sulfur-pH	96.006	1	96.006	0.273	
Nitrogen-pH	1066.673	1	1066.673	3.039	0.097
Sulfur-nitrogen-pH	149.994	1	149.994	0.427	
Error	5616.667	16	351.042		
Total	14921.833	23			

APPENDIX XIV

SULFUR LEVELS IN SEEDLING NEEDLES, % DRY WT.

DATA

pH	Treatment				
	SN	S	N	CL	CON[a]
5	0.16	0.14	0.17	0.15	0.14
	0.15	0.14	0.14	0.18	0.16
	0.15	0.14	0.14	0.18	0.13
4	0.14	0.16	0.17	0.15	--
	0.15	0.17	0.15	0.14	--
	0.15	0.15	0.15	0.18	--

[a]Control values are at pH = 5.6.

ONE-WAY ANOVA

Source of Variation	SS	DF	MS	F	P
Blocks	0.065	2			
Treatments	2.261	8	0.283	1.216	0.350
Error	3.718	16	0.232		
Total	6.044	26			

APPENDIX XIII

NITROGEN LEVELS IN SEEDLING NEEDLES, % DRY WT.

DATA

pH	Treatment				
	SN	S	N	CL	CON[a]
5	1.66	1.55	1.45	1.51	1.38
	1.54	1.36	1.43	1.67	1.48
	1.36	1.40	1.41	1.38	1.36
4	1.45	1.54	1.52	1.39	--
	1.44	1.63	1.38	1.35	--
	1.28	1.40	1.53	1.55	--

[a]Control values are at pH = 5.6.

ONE-WAY ANOVA

Source of Variation	SS	DF	MS	F	P
Blocks	3.3738	2			
Treatments	6.472	8	0.809	0.804	>0.500
Error	16.108	16	1.007		
Total	26.318	26			

APPENDIX XVI

POTASSIUM LEVELS IN SEEDLING NEEDLES, % DRY WT.

DATA

pH	Treatment				
	SN	S	N	CL	CON[a]
5	0.82	0.62	0.67	0.68	0.68
	0.75	0.78	0.66	0.78	0.76
	0.65	0.76	0.72	0.93	0.69
4	0.60	0.72	0.64	0.66	--
	0.73	0.86	0.75	0.72	--
	0.73	0.65	0.73	0.72	--

[a]Control values are at pH = 5.6.

ONE-WAY ANOVA

Source of Variation	SS	DF	MS	F	P
Blocks	2.867	2			
Treatments	2.985	8	0.373	0.728	>0.500
Error	8.206	16	0.513		
Total	14.058	26			

APPENDIX XV

PHOSPHOROUS LEVELS IN SEEDLING NEEDLES, % DRY WT.

DATA

pH	Treatment				
	SN	S	N	CL	CON[a]
5	0.17	0.16	0.18	0.18	0.20
	0.20	0.18	0.17	0.23	0.23
	0.18	0.20	0.22	0.26	0.17
4	0.16	0.17	0.21	0.20	--
	0.18	0.25	0.18	0.16	--
	0.19	0.17	0.18	0.20	--

[a]Control values are at pH = 5.6.

ONE-WAY ANOVA

Source of Variation	SS	DF	MS	F	P
Blocks	0.134	2			
Treatments	0.134	8	0.017	0.221	>0.500
Error	1.213	16	0.076		
Total					

APPENDIX XXV

EFFECTS OF LONG-TERM COLD STORAGE ON IAA LEVELS

The purpose of this experiment was to determine whether long term storage of roots at -20°C had any effect on the levels of IAA in the roots. Several loblolly pine seedlings (age approximately 1 year) were removed from their pots and their roots washed and blotted dry. The roots were removed and cut into approximately 1 cm sections. After the cut-up roots were thoroughly mixed, they were divided into six samples of 42 g each. They were replaced in a freezer at -20°C.

IAA levels were measured using the same technique outlined in the materials and methods sections. The results are presented below:

Sample	Length of Time in Cold Storage	Level IAA, g
1	24 hours	1.25×10^{-6}
2	1 week	1.09×10^{-6}
3	2 weeks	1.07×10^{-6}
4	3 weeks	0.70×10^{-6}
5	4 weeks	1.22×10^{-6}
6	5 weeks	1.06×10^{-6}

Except for Sample 4, all samples show reasonable agreement with one another. The lower level measured in Sample 4 is very likely due to a faulty reading of the pre-extraction internal standard level by the Beckman scintillation counter. The instrument was repaired the following day.

APPENDIX XXVI

COMPARISON OF IAA LEVEL MEANS FOR EXTREME M-INDICES

Two groups of roots were selected for comparison: one with an abundance of mycorrhizae (M-index 81 or greater), and one with virtually no mycorrhizae (M-index = 6, the lowest value on the M-index scale). Since the samples come from populations of different types, it cannot be assumed that the two population variances are the same. In addition to this restriction, the sample sizes are unequal. The test for significance between two population means given these two criteria follows that of Snedecor (33). The procedure is duplicated below.

IAA LEVELS, mg IAA/kg dry weight

Abundant Mycorrhizae	No Apparent Mycorrhizae
0.91	4.39
1.04	1.11
1.38	1.01
0.95	0.86
0.92	2.29
1.06	1.13
	1.02
	1.00
$\overline{X}_1 = 1.04$	$\overline{X}_2 = 1.60$
$s_1^2 = 0.03$	$s_2^2 = 1.47$
$n_1 = 6$	$n_2 = 8$
$s_1^2/n_1 = w_1 = 0.005$	$s_2^2/n_2 = w_2 = 0.18$

Since the two samples furnish two unbiased estimates of the variances, the ordinary student's t is replaced by

$$|t'| = (\overline{X}_1 - \overline{X}_2)/(s_1^2/n_1 + s_2^2/n_2)^{1/2}$$

$$|t'| = (1.04 - 1.60)/(0.005 + 0.18)^{1/2}$$

$$|t'| = 1.3$$

The student's t table is used to calculate the significance level of t' by the formula

$$t'_p = (w_1 t_1 + w_2 t_2)/(w_1 + w_2)$$

where p is the desired probability. Set p = 0.05, then

$$t_1(5 \text{ d.f.}) = 2.571 \qquad t_2(7 \text{ d.f.}) = 2.365$$

$$t'_{0.05} = (0.005 \times 2.571 + 0.18 \times 2.365)/(0.005 + 0.18)$$

$$t'_{0.05} = 2.37$$

Since t' does not exceed $t'_{0.05}$, we conclude that there are no significant differences between the means.

A test for the inequality of the two variances would be a double check for the validity of the Snedecor method. The null hypothesis is: s_1^2 and s_2^2 are from independent random samples from normal populations with the same variance. The test criterion is $F = s_1^2/s_2^2$, but in this case, s_1^2 must be chosen to correspond with the larger mean square. A Fisher table for the distribution of F when the null hypothesis is true is tabulated in Snedecor (33). With 7 degrees of freedom for the larger mean square and 5 for the smaller,

$$F = 1.47/0.03 = 49$$

From the Fisher table, $F_{0.05,7,5} = 6.87$; thus, the null hypothesis is rejected.

APPENDIX XXVII

IAA LEVELS IN WILD CARROT TISSUES

Cultures of wild carrot tissue undergoing cell proliferation were analyzed for IAA levels over a period of 28 days. The extraction was performed according to the procedure presented in this thesis, except that volumes were significantly reduced due to the small amounts of starting material (between 500-600 mg fresh tissue weight). The results presented below show the variation in the levels as they start from a maximum, drop to a minimum, and then return to the maximum at the end of the 28 days.

Day	IAA Level, ng/g fr wt.
0	51.58
7	35.65
14	25.48
21	25.03
28	51.70

APPENDIX XXVIII

BOUND IAA IN MYCORRHIZAL ROOTS

This test was performed to see if there was any evidence of bound IAA existing in mycorrhizal roots. The method of Bandurski and Schulze (38) for hydrolyzing ester and amide bound IAA was easily adaptable to the extraction procedure described in the materials and methods section of this thesis.

Several seedlings of 1 1/2-year loblolly pine were examined and the roots which displayed the most mycorrhizae were removed. A total of 51 g of roots was extracted in the usual fashion with 153 mL of methanol, with the exception that the internal standard was added to the filtrate after the first filtering step. Then, the filtrate was divided into three, 80 mL portions, with the following designations:

Portion 1 - Free IAA; no change in extraction procedure

Portion 2 - Free + ester IAA; treat with 1N NaOH for one hour at room temperature

Portion 3 - Free + ester + amide IAA; treat with 7N NaOH for 1 hour at 100°C under N_2

The change in treatments occurred immediately after the first evaporation step. After the treatments with base, Portions 2 and 3 were acidified down to the working pH of 8.0, where they resumed the course of a normal extraction.

The levels of IAA detected for the three portions are presented below.

Portion	Level IAA Detected, g
1	6.42×10^{-6}
2	7.99×10^{-6}
3	7.90×10^{-6}
Total	22.31×10^{-6}

The contribution of amide-bound IAA to the total is either negligible or nonexistent. Assuming the value in Portion 1 is the base line for free IAA in Portions 2 and 3, the contributions of the bound fractions break down as follows (neglecting the exponential rotation).

	1	2	3
Free	6.42	6.42	6.42
Ester		1.57	1.48
Amide			0.00

This indicates that the ester bound fraction of IAA makes up approximately 14% minimum of all the IAA extracted. The reason this is cited as a minimum is because one-third of the sample (Portion 1) was spared from any hydrolysis conditions. Assuming an equivalent amount of bound IAA escaped detection, the bound portion could account for as much as 19% of the total IAA in the system.

It was decided to repeat the experiment on nonmycorrhizal roots to see if a similar pattern emerged. Roots were taken from loblolly pine seedlings (approximately 6 months old) which had been planted in a sterile soil and had never been exposed to any mycorrhizae. Close examination of the roots showed no branching or forking, swelling, mantle, or discoloration: all normal characteristics of mycorrhizal roots. Again, 51 g of material were collected and the procedure duplicated as in the mycorrhizal roots.

Apart from the fact that the levels of IAA detected were significantly lower than the first batch, another surprise resulted: there was only a negligible contribution of ester-bound IAA but a significant contribution of amide-bound IAA.

APPENDIX VII

CALCULATION OF ESTIMATORS

Estimators are useful in that they replace missing or outlying data points, thereby eliminating the need to perform alternative and more complicated analyses of variance. For every estimator used, there is a corresponding reduction in the number of degrees of freedom within the system.

The formula for calculating an estimator as given by Snedecor (34) is

$$X = \frac{aT + bB - S}{(a-1)(b-1)}$$

where

a = number of treatments

b = number of blocks

T = sum of items with same treatment as missing item

B = sum of items in same block as missing item

S = sum of all observed items

If there are two missing data points, as in the case of IAA levels for N5 and N4, an iterative technique is used. The first step is to enter a reasonable value for one of the missing data, then calculate the other value.

For example, guess $X_{N4} = 1.40$, the average of the other two values. Then,

$$X_{N5} = \frac{(9)(31.81) + (3)(9.54) - 55.49}{(9-1)(3-1)}$$

$$X_{N5} = 16.21$$

C. Detected Level of IAA

FRN	=	8348
Sample size	=	1 mL
Volume injected	=	5 µL
Peak area (385)	=	9771
AMT. IAA DETECTED	=	$(9771 - 2572)/9.560 \times 10^{11}\ g^{-1}$
	=	7.53×10^{-9} g per 5 µL
	=	1.51×10^{-6} g per 1 mL

D. Adjusted Level of IAA in Roots

Correction factor	=	0.208 (from percent internal standard recovered)
Adjusted level IAA in sample	=	$1.51 \times 10^{-6}/0.208$
	=	7.26×10^{-6} g

ROOT

Amount IAA Per Fresh Weight	=	7.26×10^{-6} g IAA/39.0 g roots
	=	1.86×10^{-7} g IAA/g fresh root weight
Amount IAA Per Dry Root Weight	=	7.26×10^{-6} g IAA/9.39 g roots
	=	7.73×10^{-7} g IAA/g dry root weight

Substituting XN5 = 16.81 in the table, next try for a better estimate of XN4:

$$X_{N4} = \frac{(9)(2.81) + (3)(20.01) - 70.30}{(9-1)(3-1)}$$

$$X_{N4} = 0.94$$

With the revised estimate of X_{N4}, reestimate X_{N5}:

$$X_{N5} = \frac{(9)(31.81) + (3)(9.54) - 55.03}{(9-1)(3-1)}$$

$$X_{N5} = 16.24$$

At this point, the values converge, since recalculating X_{N4} for $X_{N5} = 16.24$ produces the same result.

APPENDIX VIII

TOTAL SHOOT HEIGHTS, cm

DATA

pH	Treatment				
	SN	S	N	CL	CON[a]
5	92.5	101.0	109.5	90.0	113.5
	119.5	99.0	93.0	88.0	105.0
	97.0	112.0	90.0	89.5	97.5
4	92.0	100.0	107.0	95.5	--
	96.5	82.0	113.0	113.0	--
	87.0	116.0	128.0	91.5	--

[a]Control values are at pH = 5.6.

ONE-WAY ANOVA

Source of Variation	SS	DF	MS	F	P
Blocks	0.389	2			
Treatments	1823.833	8	227.979	1.948	0.122
Error	1872.944	16	117.059		
Total	3697.166	26			

TWO x TWO X TWO FACTORAL ARRANGEMENT

Source of Variation	SS	DF	MS	F	P
Sulfur	7.594	1	7.594	0.065	
Nitrogen	94.010	1	94.010	0.809	
Sulfur-nitrogen	404.260	1	404.260	3.480	0.077
pH	68.344	1	68.344	0.588	
Sulfur-pH	765.010	1	765.010	6.586	0.019
Nitrogen-pH	0.510	1	0.510	0.003	
Sulfur-nitrogen-pH	75.261	1	75.261	0.648	
Error	1858.500	16	116.156		
Total	3273.489	23			

APPENDIX IX

TOTAL SHOOT WEIGHTS, g

DATA

pH	Treatment				
	SN	S	N	CL	CON[a]
5	70.0	92.5	83.5	79.5	114.0
	104.5	67.5	83.0	67.5	89.5
	90.0	93.0	70.0	60.5	96.0
4	79.5	76.0	120.0	110.5	--
	104.5	38.0	84.0	86.5	--
	93.0	110.0	114.5	77.5	--

[a]Control values are at pH = 5.6.

ONE-WAY ANOVA

Source of Variation	SS	DF	MS	F	P
Blocks	702.463	2			
Treatments	3269.018	8	408.627	1.237	0.339
Error	5283.870	16	330.242		
Total	9255.351	26			

TWO X TWO X TWO FACTORAL ARRANGEMENT

Source of Variation	SS	DF	MS	F	P
Sulfur	14.261	1	14.261	0.041	
Nitrogen	787.761	1	787.761	2.263	0.149
Sulfur-nitrogen	3.010	1	3.010	0.008	
pH	731.511	1	731.511	2.102	0.163
Sulfur-pH	1141.260	1	1141.260	3.279	0.086
Nitrogen-pH	133.010	1	133.010	0.382	
Sulfur-nitrogen-pH	29.261	1	29.261	0.084	
Error	5569.167	16	348.073		
Total	8409.239	23			

APPENDIX X

TOTAL ROOT FRESH WEIGHT, g

DATA

pH	Treatment				
	SN	S	N	CL	CON[a]
5	36.0	46.5	52.0	43.5	42.0
	54.0	27.0	26.5	39.0	51.0
	58.5	43.5	42.5	34.0	38.5
4	43.5	50.5	79.5	41.0	--
	43.0	20.5	45.0	26.0	--
	39.0	47.5	43.0	38.0	--

[a]Control values are at pH = 5.6.

ONE-WAY ANOVA

Source of Variation	SS	DF	MS	F	P
Blocks	583.796	2			
Treatments	973.463	8	121.683	1.021	0.459
Error	1907.037	16	119.190		
Total	3464.296	26			

TWO X TWO X TWO FACTORAL ARRANGEMENT

Source of Variation	SS	DF	MS	F	P
Sulfur	0.009	1	0.009	0.000	
Nitrogen	463.760	1	463.760	3.082	0.095
Sulfur-Nitrogen	33.844	1	33.844	0.225	
pH	7.594	1	7.594	0.050	
Sulfur-pH	133.010	1	133.010	0.884	
Nitrogen-pH	46.760	1	46.760	0.311	
Sulfur-nitrogen-pH	283.594	1	283.594	1.885	0.186
Error	2407.667	16	150.479		
Total	3376.239	23			

APPENDIX XXIV

CORRELATION COEFFICIENTS BETWEEN M-INDEX AND NUTRIENT LEVELS IN SEEDLING NEEDLES

Nutrient	r
B	0.082
Ca	0.145
Fe	0.078
K	0.008
Mg	0.147
Mn	0.067
Zn	0.166

APPENDIX XXIII

ALUMINUM LEVELS IN SEEDLING NEEDLES, ppm

DATA

pH	Treatment				
	SN	S	N	CL	CON[a]
5	211	193	220	248	226
	240	143	249	285	253
	274	202	150	261	246
4	236	271	283	244	--
	259	171	233	274	--
	238	254	211	253	--

[a]Control values are at pH = 5.6.

ONE-WAY ANOVA

Source of Variation	SS	DF	MS	F	P
Blocks	155.555	2			
Treatments	16800.000	8	2100.000	1.802	0.150
Error	18644.444	16	1165.278		
Total	35599.999	26			

APPENDIX XX

MANGANESE LEVELS IN SEEDLING NEEDLES, ppm

DATA

pH	Treatment				
	SN	S	N	CL	CON[a]
5	594	667	675	602	861
	750	438	511	948	617
	852	779	718	725	861
4	854	736	818	652	--
	705	544	660	701	--
	905	904	919	924	--

[a]Control values are at pH = 5.6.

ONE-WAY ANOVA

Source of Variation	SS	DF	MS	F	P
Blocks	169755.547	2			
Treatments	105866.672	8	13233.334	0.986	>0.500
Error	214844.438	16	13427.777		
Total	490466.656	26			

APPENDIX XIX

BORON LEVELS IN SEEDLING NEEDLES, ppm

DATA

pH	Treatment				
	SN	S	N	CL	CON[a]
5	32	26	28	29	25
	21	27	24	34	30
	27	30	37	42	32
4	36	28	28	35	--
	27	38	28	27	--
	33	26	30	32	--

[a]Control values are at pH = 5.6.

ONE-WAY ANOVA

Source of Variation	SS	DF	MS	F	P
Blocks	62.741	2			
Treatments	151.852	8	18.981	0.827	>0.500
Error	367.259	16	22.954		
Total	581.852	26			

APPENDIX XVII

CALCIUM LEVELS IN SEEDLING NEEDLES, % DRY WT.

DATA

pH	Treatment				
	SN	S	N	CL	CON[a]
5	0.28	0.22	0.28	0.33	0.28
	0.29	0.27	0.25	0.38	0.30
	0.30	0.25	0.34	0.38	0.27
4	0.36	0.27	0.32	0.29	--
	0.25	0.39	0.25	0.27	--
	0.29	0.26	0.25	0.34	--

[a]Control values are at pH = 5.6.

ONE-WAY ANOVA

Source of Variation	SS	DF	MS	F	P
Blocks	0.014	2			
Treatments	2.354	8	0.294	1.559	0.213
Error	3.019	16	0.189		
Total	5.387	26			

APPENDIX XVIII

MAGNESIUM LEVELS IN SEEDLING NEEDLES, % DRY WT.

DATA

pH	Treatment				
	SN	S	N	CL	CON[a]
5	0.12	0.09	0.14	0.12	0.12
	0.11	0.11	0.11	0.16	0.12
	0.14	0.11	0.14	0.13	0.10
4	0.12	0.11	0.14	0.13	--
	0.10	0.14	0.11	0.11	--
	0.12	0.12	0.10	0.13	--

[a]Control values are at pH = 5.6.

ONE-WAY ANOVA

Source of Variation	SS	DF	MS	F	P
Blocks	0.003	2			
Treatments	0.236	8	0.030	1.098	0.413
Error	0.430	16	0.027		
Total	0.669	26			